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Modulation of Peroxisome Proliferator-Activated Receptor α-Mediated Gene Transcription of Rat Peroxisomal Acyl-CoA Oxidase and Enoyl-CoA Hydratase/3-Hydroxyacyl-CoA Dehydrogenase by Members of the Nuclear Hormone Receptor Superfamily

by

Altaf Kassam ©

A thesis submitted to the faculty of Graduate Studies and research in partial fulfilment
of the requirements for the degree of Doctor of Philosophy

Department of Cell Biology

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ABSTRACT

Peroxisome proliferator-activated receptors (PPAR) are members of the nuclear hormone receptor superfamily that serve as ligand-activated transcription factors regulating the expression of genes involved in lipid metabolism (peroxisomal β-oxidation), adipogenesis, inflammation, and glucose metabolism. PPARs are activated by a diverse group of compounds termed peroxisome proliferators, which include the fibrate family of hypolipidemic drugs, eicosanoids, antidiabetic thiazolidinediones, as well as naturally occurring and synthetic mono- and polyunsaturated fatty acids. Upon binding of ligand, PPARs heterodimerize with the 9-*cis*-retinoic acid receptor (RXRα) and bind to cognate binding sequences termed peroxisome proliferator-response elements (PPRE) found in the promoter regions of target genes. PPAR-mediated gene transcription is a complex and dynamic event involving a myriad of cellular factors. This thesis examines the role of various members of the nuclear hormone receptor superfamily in modulating gene transcription by PPARs. In particular, this thesis focuses on the genes encoding the first two enzymes of the peroxisomal β-oxidation pathway, acyl-CoA oxidase (AOx) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD).

The results presented illustrate the involvement of thyroid hormone receptor α, RevErb α, constitutive androstane receptor β (CARβ), and the short heterodimer partner (SHP) in differentially modulating PPARα-mediated gene transcription from both the AOx- and HD-PPREs. Furthermore, subtype- and response element-dependent differences in gene transcription between two PPAR subtypes, α and γ, are also demonstrated. The conclusions drawn from this research further support the hypothesis that PPAR-mediated

gene transcription from PPREs is integrated with the transcriptional activities of various members of the nuclear hormone receptor superfamily, as well as with a variety of cellular coactivators and corepressors, and can be influenced by the availability of ligand so as to ensure a correct transcriptional response to extra- and intracellular stimuli from appropriate target genes. The research contained herein establishes a framework for understanding normal and dysfunctional lipid metabolism and provides an impetus for further exploration of the molecular mechanisms of PPAR-mediated gene transcription.

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LIST OF ABBREVIATIONS

ACTR	activator of the thyroid and retinoic acid receptor
AF	activation function
Amp^r	ampicillin resistance gene
AOx	acyl-CoA oxidase
AR	androgen receptor
ARC	activator-recruited cofactors
ARE	adipocyte lipid-binding protein response element
ATP	adenosine triphosphate
bp	basepair
BSA	bovine serum albumin
C-	carboxyl-
CARβ	constitutive androstane receptor β
CARLA	coactivator-dependent receptor ligand assay
CBP	CREB-binding protein
CEN	centromeric sequence
CMV	cytomegalovisrus
CoA	coenzyme A
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CPS	carbamoyl phosphate synthetase
CREB	cAMP response element-binding protein
CYP	cytochrome P450
DAX-1	dosage-sensitive sex (DSS)-adrenal hypoplasia congenita (ACH) critical region on the X, gene 1
db	diabetes gene; encodes the leptin receptor
DBD	DNA binding domain
DMEM	Dulbecco's modified eagles's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRIP	vitamin D receptor interacting protein
DR*n*	direct repeat of TGACCT-like motifs separated by *n* nucleotide(s)
DTT	dithiothreitol
EDTA	ethylenediamine tetracetic acid
EMSA	electromobility shift analysis
ER	estrogen receptor
FAD	flavin adenine nucleotide
GAL1	gene encoding the GAL1 protein; an enzyme involved in galactose metabolism
GR	glucocorticoid receptor
GRIP-1	GR-interacting protein-1
GST	glutathione-S-transferase
HAT	histone acetylase transferase
HD	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
HDAC	histone deacetylase

HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HETE	hydroxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
HIS	gene encoding the HIS3 protein; an enzymes involved in histidine synthesis
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HNF	hepatocyte nuclear factor
HRE	hormone response element
IPTG	isopropyl β-D-thiogalactoside
kDa	kiloDalton
LBD	ligand binding domain
LEU2	gene encoding the LEU2 protein; an enzymes involved in leucine biosynthesis
LTB_4	leukotriene B_4
luc	luciferase gene
LXR	liver X receptor
MAP kinase	mitogen-activated protein kinase
NAD	nicotinamide adenine dinucleotide
NCoA	nuclear receptor coactivator
NCoR	nuclear receptor corepressor
NP-40	Nonidet P-40
NUC1	human NUC1 (a PPAR isoform; also known as PPARβ, and PPARδ)
p/CIP	p300/CBP co-integrator associate protein
p/CAF	p300/CBP-associated factor
PAGE	polyacrylamide gel electrophoresis
PBP	PPARγ-binding protein
PCR	polymerase chain reaction
PGC	PPARγ-coactivator
PGK	phosphoglycerate kinase
pH	-log[H^+]
PPAR	peroxisome proliferator activated receptor
PPO	2,5-diphenyloxazole
PPRE	peroxisome proliferator-response element
RAC3	receptor-associated coactivator 3
RAR	retinoic acid receptor
RevErbα	receptor encoded on the opposite strand of the TRα gene
RIP140	receptor-interacting protein 140
RNA	ribonucleic acid
RXR	retinoid X receptor
RXRα	9-*cis* retinoic acid receptor
RZR	retinoid-like Z receptor
SDS	sodium dodecyl sulfate
SHP	short heterodimer partner receptor
SMRT	silencing mediator for RARs and THRs
SRA	steroid receptor-specific coactivator

SRC-1	steroid receptor coactivator-1
SUNCoR	small unique nuclear receptor corepressor
T3	3,3'-5-triiodothyronine
TBE	tris/borate/EDTA (buffer)
TBST	tris-buffered saline plus Tween 20
TE	tris/EDTA (buffer)
TIF2	transcriptional intermediary factor 2
TK	thymidine kinase
TR	thyroid hormone receptor
TRAM	TR-activator molecule
TRAP	thyroid hormone receptor-associated protein
TRE	TR-response element
TRP1	gene encoding the TRP1 protein; an enzymes involved in tryptophan biosynthesis
VDR	vitamin D_3 receptor
Wy-14,643	[4-Chloro-6-)2,3-xylidino)2-pyrimidinylthio] acetic acid; a peroxisome proliferator
YNBD	yeast nitrogen base with 2% glucose
15d-PGJ2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (J-Series)

SRA	steroid receptor-specific coactivator
SRC-1	steroid receptor coactivator-1
SUNCoR	small unique nuclear receptor corepressor
T3	3,3'-5-triiodothyronine
TBE	tris/borate/EDTA (buffer)
TBST	tris-buffered saline plus Tween 20
TE	tris/EDTA (buffer)
TIF2	transcriptional intermediary factor 2
TK	thymidine kinase
TR	thyroid hormone receptor
TRAM	TR-activator molecule
TRAP	thyroid hormone receptor-associated protein
TRE	TR-response element
TRP1	gene encoding the TRP1 protein; an enzymes involved in tryptophan biosynthesis
VDR	vitamin D_3 receptor
Wy-14,643	[4-Chloro-6-)2,3-xylidino)2-pyrimidinylthio] acetic acid; a peroxisome proliferator
YNBD	yeast nitrogen base with 2% glucose
15d-PGJ2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (J-Series)

CHAPTER 1

Introduction

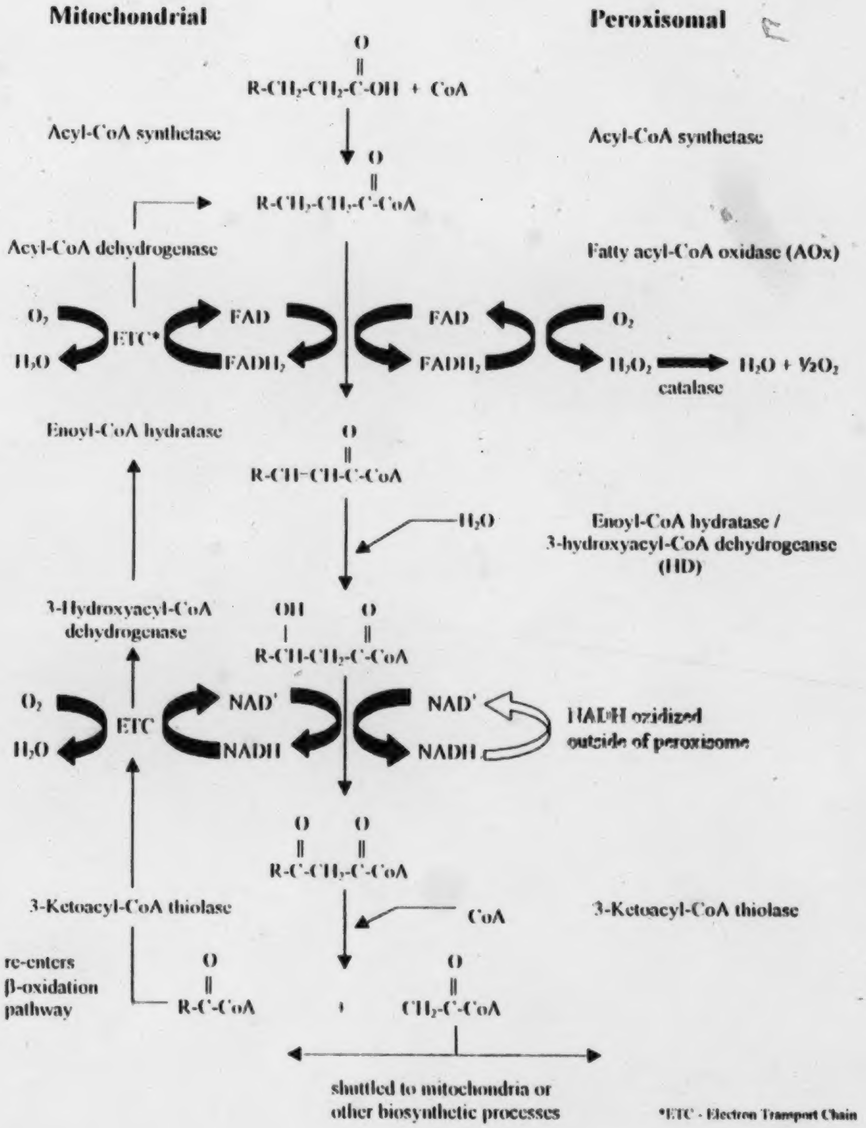
1.1 Peroxisomes

Peroxisomes are members of the microbody family of organelles which includes the glyoxysomes of plants and the glycosomes of *Trypanosomes*. They are roughly spherical in shape, varying in size from 0.2-1.0 μm in diameter, and are bound by a single unit membrane (Lazarow and Fujiki, 1985; Subramani, 1993). Mammalian peroxisomes are most abundant in the liver and kidney (Small *et al.*, 1990; Bentley *et al.*, 1993) and are involved in a myriad of biological processes which vary depending on the organism, cell type, developmental stage and cellular environment (Borst 1989; van den Bosch *et al.*, 1992; Subramani 1993). These include fatty acid β-oxidation (Lazarow and de Duve, 1976), decomposition of H_2O_2 (Lazarow and de Duve, 1976), cholesterol and plasmalogen biosynthesis (Hajra *et al.*, 1979; Tolbert *et al.*, 1981; Hajra and Bishop, 1982; Appelkvist *et al.*, 1990), bile acid synthesis (Pedersen and Gustafsson 1980, Bentley *et al.*, 1993; Hiltunen *et al.*, 1996), and the metabolism of carbohydrates, amino acids, purines and lipids (Mannaerts and van Veldhoven, 1990). To carry out the many cellular processes, peroxisomes contain a large complement of approximately 50 enzymes (Mannaerts and van Veldhoven, 1993), of which over half are involved in lipid metabolism. The importance of peroxisomes for normal human development and physiology is underscored by early infant lethality arising from human genetic disorders in which peroxisomes are absent or fail to assemble (Lazarow and Moser, 1994). Examples include Zellweger syndrome, adrenoleukodystrophy, and infantile Refsum's disease (Schutgens *et al.*, 1986; Lazarow and Moser, 1994; Singh, 1997).

Administration of a diverse group of chemical agents termed peroxisome proliferators results in a dramatic increase in the number, size, and metabolic capacity of peroxisomes (Reddy, 1990; Rao and Reddy, 1991). Peroxisome proliferators also induce a host of biochemical and morphological changes in several tissues, including hepatomegaly and tumourigenesis. In addition, hepatocarcinogenesis has also been observed after long-term exposure to peroxisome proliferators; however, this effect has been most notably observed in the liver cells of rodent species (Lock *et al.*, 1989; Bentley *et al.*, 1993).

1.2 Peroxisomal β-oxidation

Fatty acid β-oxidation can take place in either the peroxisome or the mitochondrion in animal cells (Hashimoto, 1996). In both organelles, the alkyl chains of fatty acid molecules are sequentially shortened by two carbon atoms in each oxidative cycle (Figure 1-1). The peroxisomal β-oxidation cycle consists of three core enzymes: fatty acyl-CoA oxidase (AOx), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (HD), and 3-ketoacyl-CoA thiolase (thiolase). Peroxisomes also contain catalase for the metabolism of H_2O_2 (Lehninger *et al.*, 1993; Hashimoto, 1996). In the first step of peroxisomal β-oxidation, fatty acids are activated to their acyl-CoA derivatives by an ATP-dependent acyl-CoA synthase located in the peroxisomal membrane. After transport of the acyl-CoAs across the membrane, the remaining steps are catalysed in the peroxisomal matrix (van den Bosch *et al.*, 1992). The fatty acyl-CoA is reduced to *trans*-2-enoyl-CoA by AOx, the rate-limiting enzyme. This reaction transfers electrons directly from FAD to O_2 yielding H_2O_2, which is subsequently consumed by peroxisomal catalase


Mitochondrial
Peroxisomal
R-CH₂-CH₂-C(=O)-OH + CoA
Acyl-CoA synthetase
Acyl-CoA synthetase
R-CH₂-CH₂-C(=O)-CoA
Acyl-CoA dehydrogenase
Fatty acyl-CoA oxidase (AOx)
O₂
H₂O
ETC*
FAD
FADH₂
FAD
FADH₂
O₂
H₂O₂
H₂O + ½O₂
catalase
Enoyl-CoA hydratase
R-CH=CH-C(=O)-CoA
H₂O
Enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogeanse (HD)
3-Hydroxyacyl-CoA dehydrogenase
R-CH(OH)-CH₂-C(=O)-CoA
O₂
H₂O
ETC
NAD⁺
NADH
NAD⁺
NADH
NADH oxidized outside of peroxisome
R-C(=O)-CH₂-C(=O)-CoA
3-Ketoacyl-CoA thiolase
CoA
3-Ketoacyl-CoA thiolase
re-enters β-oxidation pathway
R-C(=O)-CoA
+
CH₃-C(=O)-CoA
shuttled to mitochondria or other biosynthetic processes

*ETC - Electron Transport Chain

Figure 1-1 **Peroxisomal and mitochondrial β-oxidation Pathways**

Adapted from Principles of Biochemistry, 2nd Edition. Lehninger, AL., Nelson, DL., and Cox, MM. Worth Publishers, 1993 to produce H_2O and O_2. Next, *trans*-2-enoyl-CoA undergoes both a hydration and dehydrogenation reaction by the HD bifunctional enzyme to liberate NADH and 3-ketoacyl-CoA, which is then cleaved by thiolase to produce acetyl-CoA and a fatty acyl-CoA moiety that is two carbon atoms shorter than the original molecule (Miyazawa *et al.*, 1981). The newly formed fatty acyl-CoA re-enters the β-oxidation pathway for further oxidation (Lazarow, 1978; Hashimoto, 1987) while the acetyl-CoA is converted to carnitine esters by carnitine acyl-transferase, which are then translocated into the mitochondrial matrix by the carnitine translocase system (Hashimoto, 1996) and oxidized via the citric acid cycle. In addition, since peroxisomal β-oxidation is not directly coupled to an electron transport chain and oxidative phosphorylation, the energy released in the first oxidation step (H_2O_2 production) is lost as heat, while the energy of the second step is conserved in the form of NADH. The electrons transferred to NAD^+ in the peroxisome are reoxidized through the electron-transport chain in the mitochondrion where electron-transferring flavoproteins ultimately transfer electrons from NADH to O_2, yielding H_2O and ATP.

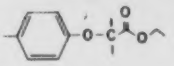
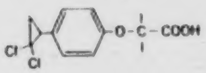
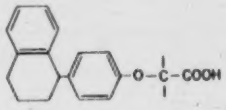
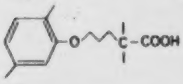
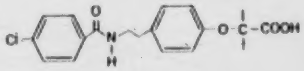
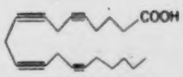
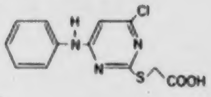
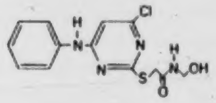
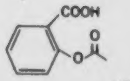
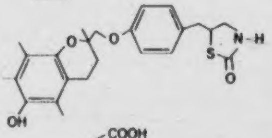
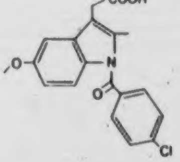
Although β-oxidation of fatty acids can occur in both peroxisomes and mitochondria, with both systems requiring fatty acyl-CoA as the initial substrate, the two systems can be distinguished according to their molecular and enzymatic properties (Lazarow, 1978; Osumi and Hashimoto, 1979; Tolbert, 1981; Lehninger *et al.*, 1993; Eaton *et al.*, 1996; Hashimoto, 1996; Mannaerts and van Veldhoven, 1996). (1) Peroxisomal β-oxidation is carnitine-independent. Therefore, very long-chain fatty acids need not be transported into the peroxisome as carnitine esters, but enter the peroxisome compartment by simple diffusion. (2) Peroxisomes preferentially oxidize medium-, long-, and very long-chain fatty acids (C_{10}-C_{30}). In contrast, mitochondria oxidize short-, medium-, and long-chain fatty acids (up to C_{18}) (Tolbert, 1981; Mannaerts and DeBeer, 1982). (3) The AOx enzymes is inactive toward substrates having acyl moieties of eight or fewer carbon atoms (Osumi, *et al.*, 1980). Instead, they are converted to carnitine esters by carnitine acyl-transferase and shuttled to the mitochondria for further oxidation (Hashimoto, 1996; Mannaerts and van Veldhoven 1996). (4) Peroxisomal β-oxidation can also use substrates that are not efficiently oxidized by mitochondria, such as unsaturated fatty acids (Osmundsen, 1982; Osmundsen and Hovik, 1988) and medium- and long-chain dicarboxylic acids (van Hoof *et al.*, 1988). (5) The activity of peroxisomes responds to various physiological conditions, the most characteristic response being the induction of β-oxidation enzymes in the presence of peroxisome proliferators (Hashimoto, 1996).

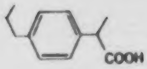
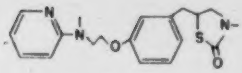
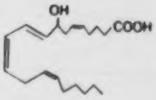
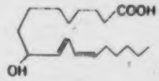
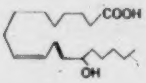
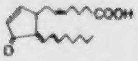
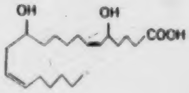
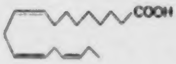
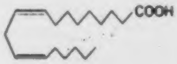
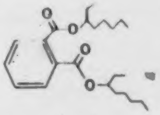
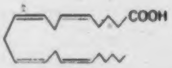
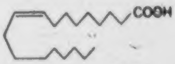
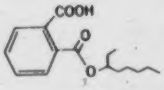
Under normal conditions, the β-oxidation of fatty acids occurs in mitochondria with the liberation of ATP (Mannaerts and DeBeer, 1982). However, under conditions in which energy balance is perturbed, such as a high fat diet or the administration of peroxisome proliferators, peroxisomal β-oxidation is induced (Lock *et al.*, 1989). It therefore appears that biotransformation via the peroxisomal β-oxidation system contributes to the oxidation of fatty acids, as well as to the detoxification of active endogenous and exogenous molecules (Mannaerts and van Veldhoven,1993; Hashimoto, 1996).

1.3 Peroxisome Proliferators

Peroxisome proliferators are a broad class of structurally unrelated xenobiotic chemicals that include herbicides, phthalate ester plasticizers, chlorinated hydrocarbons, nonsteroidal anti-inflammatory drugs, eicosanoids, thiazolidinedione antidiabetic drugs, as well as naturally occurring and synthetic mono- and polyunsaturated fatty acids (Table 1-1) (Reddy and Lalwani, 1983; Reddy and Rao, 1986; Lock *et al.*, 1989; Forman *et al.*, 1997; Kliewer *et al.*, 1997; Krey *et al.*, 1997; Lee *et al.*, 1997; Lehmann *et al.*, 1997). Surprisingly, the fibrate family of hypolipidemic drugs, which are used extensively to lower elevated plasma triglycerides and cholesterol levels in the treatment of cardiovascular disease (Reddy *et al.*, 1980), also form a class of peroxisome proliferators. Dose-response studies have shown that the potency of peroxisome proliferators varies over several orders of magnitude, with hypolipidemic agents being the strongest and plasticizers and chlorinated hydrocarbons among the weakest (Lock *et al.*, 1989). In general, peroxisome proliferators are classified as non-genotoxic carcinogens, since they fail to interact with or damage DNA either directly or indirectly via metabolic conversion as detected by a number of genotoxicity assays, including the Ames *Salmonella* mutagenecity assay (Warren *et al.*, 1980; Gupta *et al.*, 1985; Bentley *et al.*, 1987). The widespread use of peroxisome proliferators in society has led to the contamination of air, drinking water and foodstuffs, raising the concern that humans may be at a potential health risk from exposure to these chemical agents (Huber *et al.*, 1996).

Table 1-1. Examples of Peroxisome Proliferators

Common Name	Chemical Name	Structure
Fibrate hypolipidemic drugs		
Clofibrate	ethyl-α-*p*-chlorophenoxyisobutyrate	
Ciprofibrate	2-[4-[2,2-dichlorocyclopropyl] phenoxyl]-2-methyl propionic acid	
Nafenopin	2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-napthyl) phenoxy]propionic acid	
Gemfibrozil	5-2[2,5-dimethylphenoxy]2-2-dimethylpropionic acid	
Bezafibrate	2-[4-(2-[4-chlorobenzamide]ethyl)phenoxy]2-methylpropionate	
Non-fibrate hypolipidemic drugs		
ETYA	5,8,11,14-eicosatetraynoic acid	
Wy-14,643	[4-chloro-6-(2,3-xylidino)2-pyrimidinylthio] acetic acid	
BR-931	[4-chloro-6-(2,3-xylinidino)2-pyrimidinylthio] (*N*-β-hydroxyethyl)acetamide	
Non-steroidal anti-inflammatory drugs		
Aspirin (acetyl salicylic acid)	2-(acetyloxy)-benzoic acid	
Troglitazone (Rezulin)	5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione	
Rosiglitazone (BRL49653)	5-[[4[2-(methyl-2-pyridinylamino)ethoxy]phenyl] methyl]-2,4-thiazolidinedione	

Indomethacin	1-[4-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid	
Ibuprofen	methyl-4-(2-methylpropyl)phenylacetic acid	
Eicosanoids		
8S-HETE	8-hydroxy-5,9,11,14-eicosatetraenoic acid	
9-HODE	9-hydroxy-10,12-octadecadienoic acid	
13S-HODE	13-hydroxy-9,11-octadecadienoic acid	
15d-PGJ2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2	
LTB4	5,12-dihydroxy-6,7,10,14-eicosatetraenoic acid	
Unsaturated Fatty Acids		
Linolenic acid	9,12,15-octadecatrienoic acid	
Linoleic acid	9,12-octadecadienoic acid	
Arachidonic acid	5,8,11,14-eicosatetraenoic acid	
Oleic acid	9-octadecenoic acid	
Phthalates		
DEHP	Di(2-ethylhexyl)phthalate	
MEHP	Mono(2-ethylhexyl)phthalate	

1.4 Peroxisome Proliferation

Hess and coworkers (1965) and Svoboda and Azarnoff (1966) identified peroxisome proliferators through animal studies using rats administered clofibrate. Analysis of rat hepatocytes had revealed that this hypolipidemic agent caused an increase in the number, size, and metabolic capacity of peroxisomes (Paget, 1963; Hess *et al.* 1965; Svoboda and Azarnoff 1966; Lazarow and de Duve, 1976; Osumi and Hashimoto, 1978; Hawkins *et al.*, 1987). Later studies provided evidence that the increase in β-oxidation activity is accompanied by increased levels of the core enzymes of the β-oxidation pathway, AOx, HD and thiolase, primarily due to the transcriptional induction of the corresponding genes (Lock *et al.*, 1989; Hijikata *et al.*, 1990; Osumi, 1993; Reddy *et al.*, 1986). Interestingly, the same level of induction of peroxisomal catalase was not observed (Reddy and Rao, 1986, 1989).

It had been postulated that the effects of peroxisome proliferators were mediated through a receptor-based mechanism of transcriptional activation. In 1990, Issemann and Green successfully cloned the first PPAR isoform and demonstrated that, indeed, it was responsible for mediating the effects of peroxisome proliferators (Issemann and Green, 1990) by stimulating the rapid and coordinated transcriptional induction of genes encoding both the peroxisomal β-oxidation enzymes and the drug-metabolizing enzymes of the cytochrome P450 family (Poellinger *et al.*, 1992). The fact that PPARα knock-out mice fail to exhibit peroxisome proliferation upon exposure to the classic peroxisome proliferators clofibrate and Wy-14,643 further supported the claim that PPARα (NR1C1)[1] is the main mediator of the pleiotropic effects of this class of compounds. In contrast, peroxisome proliferators have been shown to have an inhibitory effect on mitochondrial-specific metabolic activities (Youssef and Badr, 1998).

1.5 Hepatomegaly and Carcinogenesis

Peroxisome proliferators have also been shown to induce a host of biochemical and morphological changes. One notable phenomena is the enlargement of the liver. Termed hepatomegaly, the growth of the liver results from both a hyperplastic (increase in cell number) and hypertrophic (increase in cell size) response. Hyperplasia is characterized by cellular proliferation due to increased DNA synthesis and a concomitant decrease in apoptosis (Rao and Reddy, 1991). On the other hand, hypertrophy results from an increase in peroxisomal volume (Meyer and Afzelius, 1989). It has been demonstrated that long-term exposure to peroxisome proliferators invariably leads to the formation of hepatocellular tumours in rodent species. However, exposure to peroxisome proliferators has not been conclusively shown to induce hepatomegaly or hepatocarcinogenesis in non-rodent species, including humans, even though peroxisome proliferators can activate both human and murine PPARα in cell-based transactivation assays (Bentley *et al.*, 1993; Bieri and Lhuguenot, 1993; Cattley *et al.*, 1998). This lack of hepatic carcinogenic response may be attributed to reduced levels of PPARα in humans (Bell *et al.*, 1998; Cattley *et al.*,

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[1] Section 1.8 Nuclear Hormone Receptor Superfamily offers an explanation for the unified nomenclature for nuclear hormone receptors.

1998; Palmer *et al.*, 1998), which are 10-fold lower in human liver than in rat liver. In addition, structural polymorphisms in human PPARα may render this form less responsive to peroxisome proliferators and/or to inducing a proliferative effect (Palmer *et al.*, 1998). Furthermore, an alteration of the PPRE sequence in the human AOx gene promoter may also partly explain the relative unresponsiveness of humans to PPARα ligands (Woodyatt *et al.*, 1999).

1.6 Oxidative Stress

A number of models have been proposed to account for the mechanisms by which peroxisome proliferators induce hepatocarcinogenesis (Rao and Reddy 1991; Bentley *et al.*, 1993; Peters *et al.*, 1997; Christiansen *et al.*, 1998). Reddy and colleagues (1980) first proposed that since the three core enzymes of the β-oxidation pathway are significantly induced, with only a minor increase in catalase activity (Lazarow and de Duve 1976; Cohen and Grasso, 1981; Reddy and Lalwani, 1983), the increase in the metabolic capacity of the β-oxidation system causes an overproduction of H_2O_2 within the cell that cannot be sufficiently converted to H_2O and O_2. The accumulation of H_2O_2 leads to increased formation of highly reactive oxygen radicals (Elliot *et al.*, 1986) that can damage DNA directly. In addition, administration of peroxisome proliferators has been shown to reduce the efficiency of the antioxidant defence system involving glutathione peroxidase and glutathione-S-transferase, which normally functions to inactivate peroxides (Furukawa *et al.*, 1985; Foliot *et al.*, 1986; Elliot and Elcombe, 1987; Rao and Reddy 1991; Reddy and Chu, 1996). In support of this, co-administration of the antioxidant ethoxyquin with the peroxisome proliferator, ciprofibrate, inhibits hepatic tumourigenesis by minimizing the increase in oxygen radical formation (Rao *et al.*, 1984).

1.7 Tumour Promotion

An alternative hypothesis linking peroxisome proliferators and hepatocarcinogenesis involves tumour promotion. Several peroxisome proliferators have been tested in assays for liver tumour initiation and were found to lack tumour-initiating activity (Williams *et al.*, 1987; Cattley *et al.*, 1989). However, peroxisome proliferators have been shown to stimulate the growth of preneoplastic lesions initiated by known carcinogens (Marsman, 1988; Green, 1992; Bentley, 1993). The increase in size of preneoplastic lesions has been attributed to an induction of DNA synthesis and mitotic activity (Moody and Reddy, 1978). Recent evidence has indicated that peroxisome proliferators prevent apoptosis in preneoplastic lesions by modulating PPAR-mediated *bcl-2* expression (Schulte-Hermann *et al.* 1991; Christiansen *et al.*, 1998). Further evidence for the involvement of PPARs in hepatocarcinogenesis comes from analysis of PPARα knock-out mice, which fail to develop hepatocellular neoplasms upon exposure to the peroxisome proliferator, Wy-14,643 (Peters *et al.*, 1997). Although the mechanism of carcinogenicity of peroxisome proliferators remains incompletely known, it likely involves PPARα and requires the continuous administration of peroxisome proliferators to induce hepatomegaly and promote tumourigenesis (Bentley, 1993).

1.8 Nuclear Hormone Receptor Superfamily

The nuclear hormone receptor superfamily[2] is divided into the steroid receptor family and the thyroid/retinoid/vitamin D_3 (or nonsteroidal) receptor family. Each type of receptor constitutes a subfamily (Table 1-2). Each receptor subtype represents the products of individual genes, and receptor isoforms represent the products of alternative gene splicing or promoter usage or both.

1.8.1 Thyroid/Retinoid/Vitamin D_3 Receptor Family

Whereas the steroid hormone receptors typically bind to response elements as homodimers, receptors of the thyroid/retinoid/vitamin D_3 family can be further classified into two subgroups based on their DNA binding properties: (1) receptors that bind as monomers, where the DNA binding domain of the receptor binds with high affinity to a single extended TGACCT half-site (Wilson, 1992); and (2) receptors that bind as heterodimers with the common partner, 9-*cis*-retinoic acid receptor (RXRα) (NR2B1) (Bugge *et al.*, 1992; Kliewer, 1992; Wahlstrom *et al.*, 1992). For this last group of receptors, the response elements are complex, often being composed of two or more TGACCT half-sites organized as either direct, inverted or everted repeats (Brent *et al.*, 1992; Farsetti *et al.*, 1992; Williams *et al.*, 1992; Mangelsdorf and Evans, 1995) with variations in the relative spacing of the half-site motifs (Table 1-3). In the case of direct

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[2] The discovery and identification of nuclear hormone receptors never followed any common nomenclature or basic naming scheme. Therefore, identical receptors cloned in different species or by different groups were given unrelated names. Recently, a unified nomenclature system for the nuclear hormone receptor superfamily has been adopted (The Nuclear Receptor Nomenclature Committee) (see Table 1-2)

Table 1-2. Members of the Nuclear Hormone Receptor Superfamily

Receptor	Acronym	Subfamily*	Subtypes	Isoforms
Steroid Family				
Progesterone Receptor	PR	3C	–	–
Androgen Receptor	AR	3C	–	–
Glucocorticoid Receptor	GR	3C	–	–
Mineralocorticoid Receptor	MR	3C	–	–
Estrogen Receptor	ER	3A	α, β	–
Thyroid/Retinoid/Vitamin D_3 Family				
Peroxisome Proliferator-Activated Receptor	PPAR	1C	α, γ, δ (or β)	γ1, γ2, γ3
Retinoic Acid Receptor	RAR	1B	α, β, γ	α1, α2, β1, β2, β3, β4, γ1, γ2
Vitamin D_3 Receptor	VDR	1I	–	–
Retinoid X Acid Receptor	RXR	2B	α, β, γ	α1, α2, β1, β2, γ1, γ2
Thyroid Hormone Receptor	TR	1A	α, β	α1, β1, β2
RevErb	–	1D	α, β	–
Retinoid Z Receptor	RZR	1F	α, β	
(a.k.a retinoid orphan receptor)	ROR	1F	α, β, γ	α1, α2, α3, α4
Farnesoid X Receptor	FXR	1H	–	–
Hepatocyte Nuclear Factor-4	HNF-4	2A	α,β,γ	α1, α2, α4
Chicken Ovalbumin Upstream Promoter Transcription Factor (I and II)	COUP-TF	2F	α,β,γ	–
Liver X Receptor	LXR	1H	α, β	–
Constitutive Androstane Receptor	CAR	1I	α, β	–
Short Heterodimer Partner	SHP	0A	–	–

* Unified Nomenclature System for the Nuclear Hormone Receptor Superfamily (1999)

In this system, the gene subfamilies are designated by Arabic numerals, groups by capital letters, and individual genes by a second set of Arabic numerals. Receptor isoforms arising from the same gene by alternative promoter usage or differential splicing are designated by a lowercase letter at the end of the name

Table 1-3. Nuclear Hormone Receptor Response Elements

Binding Sites	Motif	Receptors
Consensus Steroid Response Elements		
AGAACAnnnTGTTCT	GRE	AR/AR, GR/GR, MR/MR, PR/PR
AGGTCAnnnTGACCT	ERE	ER/ER
Consensus Direct Repeats		
TGACCTnTGACCT	DR1	RXR/RXR, PPAR/RXR RAR/RXR, COUP-TF/RXR
TGACCTnnTGACCT	DR2	RAR/RXR
TGACCTnnnTGACCT	DR3	VDR/RXR
TGACCTnnnnTGACCT	DR4	TR/RXR
TGACCTnnnnnTGACCT	DR5	RAR/RXR

Peroxisome Proliferator Response Elements

AOx

TGACCTtTGTCCT

RevErbα Response Element (Monomer)

TGACC(T/C)ACATT

TRE (palindrome)

AGGTCAtTGACCT

β-RARE

TGAACTttcggTGAACC

CYP4A6

TCACTTtTGCCCT

HD

TCTCCTtTGACCTatTGAACTaTTACCT

DR2

TGACC(T/C)gaTGACC(T/C)ACATT

DR4

AGGTCAtttcAGGACA

GRE - glucocorticoid response element
ERE - estrogen response element
AOx - acyl-CoA oxidase
HD - enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
TRE - thyroid hormone receptor response element
β-RARE - retinoic acid receptor β2 response element
CYP4A6 - cytochrome P450 fatty acid ω-hydroxylase
n - any nucleotide

repeats, spacing with one nucleotide (DR1) is typically used by PPAR/RXR heterodimers and RXR homodimers, while direct repeats with 3,4, or 5 nucleotide spacing (DR3, DR4, DR5) are the preferred target sites for heterodimers of RXR with vitamin D3 receptor, TRs, and retinoic acid receptor, respectively (Kliewer *et al.*, 1992a). However, some members of the nuclear hormone receptor superfamily exhibit binding promiscuity that enables them to bind to direct repeats with variable spacing, albeit at lower efficiency than binding to their cognate binding sites (Naar *et al.*, 1991; Umesono *et al.*, 1991; Tsai and O'Malley, 1994). For example, COUP-TF I (NR2F1) homodimers can bind to direct and inverted repeats with spacing between 1 and 10 nucleotides (Cooney *et al.*, 1992) Binding promiscuity confers crosstalk among nuclear hormone receptor signaling pathways and adds a further layer of complexity onto the molecular mechanisms of transcriptional regulation (Umesono *et al.* 1991).

1.9 Structural and Functional Domains

The nuclear hormone receptors are modular in structure, composed of four functional domains (A/B, C, D, and E/F) (Lemberger *et al.*, 1996), which are classified on the basis of sequence homology and functional conservation (Mangelsdorf and Evans 1995; Nuclear Receptors Nomenclature Committee, 1999). The amino-terminal A/B domain is highly variable in terms of length and sequence, and contains an autonomous activation function (AF1) domain that plays an important role in gene expression (Section 1.9.2). The C domain represents the highly conserved DNA binding domain (DBD), which consists of two zinc finger motifs, each comprised of four cysteine residues coordinated with a zinc atom via their sulphur residues. This results in the formation of a globular tertiary structure that enables the receptor to bind to specific DNA response elements in the promoter regions of target genes (Evans, 1988; Umesono and Evans, 1989; Luisi *et al.*, 1991; Gronmeyer, 1992; Parker, 1993; Mangelsdorf *et al.*, 1995; Meier, 1997; Forman *et al.*, 1998; Keightley, 1998). DNA binding specificity is also influenced by the P-box amino acids located at the carboxy-terminal end of the first zinc finger. Members of the PPAR/TR/RAR subfamily and some orphan nuclear receptors share an identical P-box motif of CEGCKG. In addition, the amino acids between the first and second cysteine residues of the second zinc finger comprise the D-box, which is involved in receptor dimerization (Kumar and Thompson, 1999). Unlike members of the TR and RAR subfamily that have five or six amino acids, PPARs have only three amino acids that are important in directing the spatial orientation of PPAR and its dimerization partner, RXR. The D region, also known as the hinge region, confers spatial flexibility, allowing conformational changes within the receptor upon DNA and ligand binding. The flexibility of the hinge region is critical, as it permits the DNA binding domain to rotate 180°, enabling some receptors to bind as dimers to both direct and inverted response elements (Glass, 1994) Furthermore, the hinge region contains nuclear localization sequences, mediates interactions with corepressor proteins, and participates in DNA binding (Shibata *et al.*, 1997b; Jackson, 1997). The E/F domain is a complex domain that functions primarily as the ligand binding domain (LBD). It contains a ligand-dependent transactivation function (AF2) domain (Section 1.9.3), as well as houses largely uncharacterized subdomains involved in transcriptional repression, dimerization, and nuclear localization (Kumar and Thompson, 1999; Giguère, 1999). The ligand binding domains of nuclear hormone receptors are very similar, comprised of eleven to thirteen highly conserved α-helices and one or more conserved antiparallel β-sheets, arranged into three layers that form the hydrophobic pocket of the ligand binding site (Torchia *et al.*, 1998; Kumar and Thompson, 1999).

1.9.1 Ligand Binding Sites of PPARs

X-ray crystallographic studies on the human apo-PPARγ-LBD and apo-PPARδ-LBD have illustrated that the PPARs contain an unusually large ligand binding pocket with a volume of approximately 1300 Å^3. It has been estimated that when bound, ligands occupy from 30 to 40% of the binding cavity volume, in contrast to most other nuclear receptors, such as the TRs in which ligands occupy close to 90% of the cavity volume of a significantly smaller, 600 Å^3 binding pocket (Wagner *et al.*, 1995). The large binding cavity of PPARs offers an explanation for their ability to bind a large repertoire of structurally diverse compounds, albeit with low affinity (Xu *et al.*, 1999; Nolte *et al.*, 1998), rather than a single, high affinity ligand typical of most nuclear hormone receptors.

1.9.2 Activation Function-1 (AF1) Domain

The AF1 domain, unlike the AF2 domain (Section 1.9.3), is conventionally thought to be constitutively active in a ligand-independent manner by molecular mechanisms that are poorly understood (Glass *et al.*, 1997; Gelman *et al.*, 1999). Circular dichroism and nuclear magnetic resonance studies have shown that the AF1 domain is rich in acidic amino acid residues (Dahlman-Wright *et al.*, 1995) capable of forming one or more α-helical structures *in vitro*. Recent studies involving a number of nuclear hormone receptors have indicated that specific serine residues in the AF1 region are subject to cyclin-dependent kinase (CDK)- and mitogen-activated protein (MAP)-kinase-induced phosphorylation. Depending on the receptor type, phosphorylation can either activate or repress transcription (Hammer, 1999; Tremblay, 1999). Insulin stimulation has also been shown to activate PPARα by phosphorylation of its AF1 domain by MAP kinase (Juge-Aubry *et al.*, 1999). It is widely believed that phosphorylation promotes the dissociation of corepressor proteins, consequently permitting the recruitment of coactivators. Indeed, it has been demonstrated that the AF1 domain can bind to the p160 family of coactivators (Oñate *et al.*, 1998; Webb *et al.*, 1998; Ma, 1999) as well as p300. In the case of ERβ (NR3A2), coactivators (Section 1.15.1) such as SRC-1 are recruited to AF1 independently of the typical ligand-dependent AF2 transactivation domain (Tremblay *et al.*, 1999). Furthermore, phosphorylation of the AF1 domain of steroidogenic factor 1 (SF-1) allows for recruitment of the coactivator GRIP-1 to its LBD (Hammer *et al.*, 1999). These observations imply the existence of intramolecular communication between the AF1 and AF2 domains, whereby phosphorylation of the AF1 domain propagates conformational changes to the LBD, facilitating coactivator recruitment (Freedman, 1999b). However, phosphorylation can also have an inhibitory effect, as in the case of MAP kinase-induced phosphorylation of PPARγ (Hu *et al.*, 1996), which significantly down-regulates PPARγ transcriptional activity by reducing its ligand-binding affinity and modifying its interactions with coactivators and corepressors (Shao *et al.*, 1998; Hu *et al.*, 1996).

It has also been reported that the AF1 domain can form functional, physical interactions with components of the basal transcriptional machinery such as TFIID (Ford *et al.*, 1997). More recently, two-hybrid screens for AF1 interacting proteins have led to the isolation of steroid receptor-specific coactivators (SRA), which are novel RNA species speculated to form a ribonucleoprotein transcriptional scaffold through which SRC-1 is recruited to steroid receptors (Freedman, 1999a; Lanz *et al.*, 1999).

1.9.3 Activation Function-2 (AF2) Domain

Mutational analysis of the estrogen receptor (ER) revealed a region within its LBD necessary for transactivation (Danielian *et al.*, 1992). This region is commonly known as the activation function (AF)-2 domain. X-ray crystallography data of various nuclear hormone receptors has illustrated the dynamic nature of the LBD, which undergoes significant structural changes between the unbound, agonist-, and antagonist-bound states (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995; Brzozowski *et al.*, 1997; Nolte *et al.*, 1998). Ligand binding stimulates nuclear hormone receptor activity by inducing a conformational change within the AF2 domain that facilitates interactions between the nuclear hormone receptor and coactivators. The LBD of nuclear hormone receptors is well conserved and generally consists of 12 α-helices with a conserved β-turn between α-helices H5 and H6 (Moras and Gronemeyer, 1998; Nolte *et al.*, 1998; Torchia *et al.*, 1998), while α-helices H3, H4, H5 and H12 form the hydrophobic pocket that binds ligand. Structural analysis of the LBDs of RAR, RXR, ER, TR and PPAR illustrate that upon binding of ligand, the AF2 domain becomes repositioned so that it folds up against the ligand binding pocket, forming a "charge clamp" that holds the ligand in place, while also exposing a surface for coactivator recognition and binding (Moras and Gronemeyer, 1998; Darimont *et al.*, 1998; McInerney *et al.*, 1998). Deletions and mutations involving the H12 α-helix eliminate ligand-dependent AF2 transactivity, underscoring the importance of this α-helix in nuclear hormone receptor transactivity (Tone *et al.*, 1994; Jurutka *et al.*, 1997). In contrast, antagonist-bound ER causes H12 to become partially buried within the ligand binding pocket, precluding the residues needed for coactivator recruitment and transcriptional activation (Brzozowski *et al.*, 1997).

1.10 Orphan Nuclear Receptors

Classic members of the nuclear hormone receptor superfamily were originally cloned on the basis that known hormones (steroids) bound specific receptors that mediated their physiological effects (Evans, 1988; Jensen, 1996). However, with the advent of molecular biology, low-stringency screening of cDNA libraries using conserved DNA binding domain fragments led to the cloning of a plethora of cDNAs encoding nuclear hormone receptors. Of the 70 known nuclear hormone receptors, putative ligands have only been identified for approximately half of them (Kliewer *et al.*, 1999). The remaining receptors with unknown ligands are collectively referred to as orphan nuclear receptors. This large class of receptors is implicated in a wide variety of biological processes that continue to expand our understanding of nuclear hormone receptor biology. The vast majority of orphan nuclear receptors possess all the functional domains typical of nuclear hormone receptors. However, variability exists in that some receptors have short A/B domains that lack an AF1 domain, while others such as RevErbα (NR1D1) lack an AF2 domain (Harding and Lazar, 1993), Furthermore, receptors such as SHP (NR0B2) and DAX-1 (NR0B1) are found to be missing a typical DNA binding domain. However, in the absence of specific, well characterized functional domains, such orphan receptors may still contain novel functional domains that allow them to bind DNA and ligands while also interacting with cofactors (Zazopoulos *et al.*, 1997). This is exemplified by the DAX-1 receptor, which uses its unique amino-terminal domain to bind to DNA hairpin loop structures (Zazopoulos *et al.*, 1997).

In order to better understand the physiological role of orphan receptors in health and disease, a number of research groups have been devising strategies to identify orphan receptor ligands through a screening process known as "reverse endocrinology" (Blumberg and Evans 1998; Gustaffson, 1999; Kliewer *et al.*, 1999; Schapira *et al.*, 2000; Williams, 2000). In some cases, ligand identification has been based on the hypothesis that ligand binding to nuclear hormone receptors induces an interaction with coactivator proteins *in vitro* (Krey *et al.*, 1997; Desvergne *et al.*, 1998). The ligand-dependent interaction of SRC-1 with PPARγ led to the development of a coactivator-dependent receptor ligand assay (CARLA), which has enabled the identification of *bona fide* ligands for PPARs from *Xenopus* (Krey *et al.*, 1997). Technological advances in instrumentation have also led to the development of new cell-based assays for measuring ligand binding and receptor activation (Williams, 2000), as well as increasing the speed with which putative ligands are discovered.

1.11 Nuclear Hormone Receptor Response Elements

Nuclear hormone receptors function to regulate transcription by binding to specific DNA sequences, termed response elements, located in the promoter regions of target genes (Umesono and Evans, 1989; Glass, 1994; Mangelsdorf and Evans, 1995; Keightley, 1998). These response elements are comprised of two copies of consensus hexameric motif TGACCT (Glass, 1994; Schoonjans *et al.*, 1996; Latruffe and Vamecq, 1997) or variations thereof, configured in tandem as direct, inverted, everted or palindromic repeats (Mangelsdorf and Evans, 1995). Specificity of binding by nuclear hormone receptors is dictated by the orientation and spacing of the repeats, as well as by both the core consensus sequence and sequences adjacent to the TGACCT motif (Umesono *et al.*, 1991; Mader *et al.*, 1993; Castelein *et al.*, 1997; IJpenberg *et al.*, 1997; Juge-Aubry *et al.*, 1997; Olson and Koenig, 1997; Osada *et al.*, 1997)

1.11.1 Peroxisome Proliferator Response Elements

PPARs heterodimerize with the 9-*cis* retinoic acid receptor (RXRα) and bind to specific response elements termed peroxisome proliferator-response elements (PPRE) comprised of direct tandem repeats of the consensus hexameric motif TGACC(T/C) separated by one nucleotide, referred to as DR1 spacing (Kliewer *et al.*, 1992b; Gearing *et al.*, 1993; Keller *et al.*, 1993; Chu *et al.*, 1995). PPAR/RXR heterodimers favour PPREs in which the separating nucleotide is either an A or T (Palmer *et* 1995; Johnson *et al.*, 1996; Juge-Aubry *et al.*, 1997). Although the PPREs function primarily to bind PPARs, they also can be bound by other nuclear hormone receptors in DNA binding assays and can differentially modulate PPAR function in transient transfection assays (Miyata *et al.*, 1993; Miyata *et al.*, 1996; Winrow *et al.*, 1994, Winrow *et al.*, 1998). The convergence of various nuclear hormone receptor signaling networks with the PPAR signaling pathway has been demonstrated for COUP-TF I (NR2F1) (Miyata *et al.*, 1993), HNF-4 (NR2A1) (Winrow *et al.*, 1993), LXRα (Miyata *et al.*, 1996), and RZRα (NR1F1) (Winrow *et al.*, 1998). Furthermore, other signaling pathways such as those involving mitogen-activated protein (MAP) kinase can also target nuclear hormone receptors directly and modify their activity.

1.11.2 PPREs of the Genes Encoding β-oxidation Enzymes

PPREs have been found in the promoters of a number of target genes including those encoding peroxisomal acyl-CoA oxidase (AOx) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD). The PPREs of AOx and HD were first identified by deletional and mutational analysis of their promoter regions, followed by transient transfection in the peroxisome proliferator-responsive Reuber rat hepatoma cell line, H4IIEC3 (Osumi *et al.*, 1991; Zhang *et al.*, 1992, 1993). However, the HD-PPRE is unique in that it contains four naturally occurring half-sites (Chu *et al.*, 1995). These four imperfect TGACC(T/C) motifs constitute a unique binding site consisting of two DR1 elements separated by two nucleotides, hence forming a juxtaposed DR2 element (Chu *et al.*, 1995) (Table 1-3). The integrity of the DR1 repeat of the AOx- and HD-PPREs is essential for PPAR/RXR binding *in vitro* and for peroxisome proliferator-responsiveness *in vivo* (Issemann *et al.*, 1993; Miyata *et al.*, 1993; Chu *et al.*, 1995). In addition to the

AOx- and HD-PPREs, related PPREs have been found in a number of other peroxisome proliferator-responsive genes including those encoding ω-hydroxylases (*CYP4A1* and *CYP4A6*) (Muerhoff *et al.*, 1992; Aldridge *et al.*, 1995), fatty acyl CoA-sythetase (Schoonjans *et al.*, 1995), malic enzymes (Castelein *et al.*, 1994), liver fatty acid binding protein (Issemann *et al.*, 1992), and lipoprotein lipase (Schoonjans *et al.*, 1996). It is becoming increasingly apparent that sequences flanking the PPRE are also important for directing the binding of, and maintaining the conformation of PPAR/RXR heterodimers to the DNA (Palmer *et al.*, 1995; Castelain *et al.*, 1997; IJpenberg *et al.*, 1997; Juge-Aubry *et al.*, 1997; Osada *et al.*, 1997). These adjacent residues can also modulate gene activation by regulating the specificity of nuclear hormone receptors that bind the PPREs (Palmer *et al.*, 1995).

Analysis of both natural and synthetic PPREs has demonstrated heterodimer binding polarity, with PPAR binding to the 3' half-site and RXR binding to the 5' half-site (Chu *et al.*, 1995; IJpenberg *et al.*, 1997; Juge-Aubry, 1997). This is in contrast to the binding polarity of other RXRα heterodimeric complexes, such as TRα/RXRα, where RXRα occupies the distal half-site. This differential polarity can be explained by intrinsic differences within the specific response elements, which provide a more stable binding interface for specific combinations of nuclear hormone receptor heterodimers in different polar orientations. The unique D-box of PPARs can also exert influence on binding polarity.

1.12 The PPARs

PPARs were initially cloned from mouse in a genetic screen for novel members of the nuclear hormone receptor superfamily (Issemann and Green, 1990) and were shown to be activated by peroxisome proliferators in cell-based transfection assays. The PPARs belong to the thyroid/retinoid/vitamin D3 receptor superfamily, members of which recognize response elements consisting of direct repeats of the TGACC(T/C) motif separated by one base pair (Schoonjans *et al.*, 1996; Latruffe and Vamecq, 1997). There are three PPAR subtypes, α, γ, and δ (also known as PPARβ, NUC1 and FAAR), of which the γ subtype contains three isoforms, 1, 2 and 3. The three PPAR subtypes show distinct tissue patterns of expression and have been identified in a number of species, including human (Schmidt *et al.*, 1992; Sher *et al.*, 1993), rat (Gottlicher *et al.*, 1992), mouse (Chen *et al.*, 1993; Issemann and Green, 1990; Tontonoz *et al.*, 1994; Zhu *et al.*, 1993), guinea pig (Bell *et al.*, 1998), hamster (Aperlo *et al.*, 1995), salmon (Ruyter *et al.*, 1997) and *Xenopus* (Dreyer *et al.*, 1992). In addition, the three PPAR subtypes differ in their ligand binding profiles, each preferentially binding a specific subset of natural and synthetic ligands (Issemann and Green, 1990; Kliewer *et al.*, 1995; Forman *et al.*, 1997). Together, the PPARs regulate the transcription of genes involved in a multitude of metabolic processes that include lipid metabolism, adipogenesis, inflammation, glucose metabolism, development, differentiation, and cancer (Dreyer *et al.*, 1992; Green and Wahli, 1994; Devchand *et al.*, 1996; Schoonjans *et al.* 1996; Latruffe and Vamecq, 1997; Ribon *et al.*, 1998; Ricote *et al.*, 1998; Deeb *et al.*, 1998; Wu *et al.*, 1998; Desvergne and Wahli, 1999; He *et al.*, 1999).

1.12.1 PPARα

PPARα (NR1C1) is predominantly expressed in liver, heart, kidney, muscle and brain tissue (Braissant *et al.*, 1996; Auboeuf *et al.*, 1997). PPARα is activated by both naturally occurring and synthetic long- and very long-chain fatty acids, the fibrate family of hypolipidemic drugs, eicosanoids, leukotriene B4 (LTB4), thiazolidinediones (TZD), and a variety of peroxisome proliferators (Forman *et al.*, 1997; Kliewer *et al.* 1997; Krey *et al.*, 1997; Schoonjans *et al.*, 1997). The PPARα subtype is responsible for regulating the expression of genes essential for fatty acid β-oxidation; detoxification of several xenobiotics; lipoprotein, cholesterol, and triglyceride metabolism; and ketogenesis. PPARα also plays a role in controlling inflammation. The potent chemotactic inflammatory eicosanoid, LTB4 binds to and activates PPARα, inducing the transcription of enzymes of the ω- and β-oxidation pathways that eventually catabolize LTB4 (Devchand *et al.*, 1996). In agreement, activation of PPARα with clofibrate accelerates the catabolism of LTB4 in granulocytes and macrophages (von Schacky *et al.*, 1993; Couve *et al.*, 1992). Taken together, PPARα modulates the expression of inflammatory cytokines and inflammation markers, exerts anti-inflammatory and pro-apoptotic activities, and is implicated in atherosclerosis, cell differentiation, obesity, carcinogenesis and cell cycle control (Gonzalez, 1997; Aoyama *et al.*, 1998; Chinetti *et al.*, 1998; Ricote *et al.*, 1998; Peters *et al.*, 1998; Gelman *et al.* 1999; Medh, 1999; Pineda-Torra *et al.*, 1999). Analysis of PPARα knock-out mice reveals that these mice fail to exhibit the hallmarks of the peroxisome proliferator response (Lee *et al.*, 1995; Costet *et al.*, 1998), have a decreased propensity for the onset of hepatocarcinogenesis, have increased circulating levels of cholesterol, and sustain a prolonged inflammatory response in response to treatment with fibrates (Peters *et al.*, 1997).

1.12.2 PPARγ

PPARγ (NR1C3) has been the most extensively studied PPAR subtype. Whereas PPARα functions in the catabolism of fatty acids, PPARγ is involved in lipid storage. The PPARγ gene is transcribed into three PPARγ mRNA species, PPARγ1, PPARγ2, and PPARγ3, which are derived by alternative splicing and promoter usage (Fajas *et al.*, 1997; Zhu *et al.*, 1995). PPARγ2 differs from PPARγ1 and PPARγ3 by an additional 30 amino acids at its amino-terminal (Tontonoz *et al.*, 1994; Camp and Tafuri, 1997; Fajas *et al.*, 1997, 1998). It has been demonstrated that PPARγ1 and PPARγ2 are phosphorylated by MAP-kinase signaling pathways, which decreases their transcriptional activity by abrogating ligand-binding and/or preventing conformational changes that modulate cofactor affinity and interactions with the basal transcription machinery (Arias *et al.*, 1994; Camp and Tafuri, 1997; Adams *et al.*, 1997; Shao *et al.*, 1998). PPARγ1 is ubiquitously expressed. PPARγ2 is expressed mainly in fat cells, with moderate expression in the large intestine and monocyte precursors (Lemberger *et al.*, 1996). PPARγ1 and PPARγ3 appears to be the major subtypes expressed in macrophage foam cells (Ricote *et al.*, 1998). Foam cells are cholesterol-laden macrophages that are implicated in atherosclerotic plaque formation (Willson *et al.*, 2000). The conversion of macrophages to foam cells involves the uptake of oxidized low density lipoprotein particles via the CD36 transporter, whose expression is regulated by PPARγ (Ricote *et al.*, 1998; Tontonoz *et al.*, 1998; Willson *et* *al.*, 2000).

The PPARγ2 isoform was initially discovered as a key determinant of adipogenesis (Tontonoz *et al.*, 1994; Wahli *et al.*, 1995), where ectopic expression of PPARγ2 in preadipocytes and fibroblasts converted them to mature adipocytes (Spiegleman *et al.*, 1996; Schoonjans *et al.*, 1997; Gustaffson, 1998). The adipocyte-specific genes that are regulated by PPARγ2 include lipoprotein lipase, acyl-CoA synthase, fatty acid transport protein, phosphoenoylpyruvate carboxykinase, and the insulin-dependent glucose transporter, GLUT4 (Tontonoz *et al.*, 1995; Schoonjans *et al.*, 1996; Martin *et al.*, 1997, Motojima *et al.*, 1998; Wu *et al.*, 1998; Kersten *et al.*, 2000), which are involved in coordinating the uptake, metabolism and storage of fatty acids. Furthermore, PPARγ2 decreases the expression of adipocyte-derived leptin signaling molecule, which governs energy intake and energy usage and serves to further modulate the adipogenic effect (Kallen and Lazar, 1996). PPARγ2 is also important for the control of thermogenesis in brown fat through its regulation of uncoupling protein 1 (UCP-1) in the mitochondria (Puigserver *et al.*, 1998; Guardiola-Diaz *et al.*, 1999). Ligands for PPARγ include naturally occurring prostaglandin metabolites such as 15d-PGJ2, synthetic ligands such as TZDs which are insulin sensitizers used in the treatment of diabetes (Kliewer *et al.*, 1995; Lehmann *et al.*, 1995; Berger *et al.*, 1996; Forman *et al.*, 1997), and certain nonsteroidal anti-inflammatory drugs (NSAIDs) (Lehmann *et al.*, 1997). The central role of PPARγ in fat cell differentiation, and its activation by drugs that modulate insulin sensitization, have directly linked PPARγ to obesity and non-insulin dependent diabetes mellitus (Deeb *et al.*, 1998; Ringel *et al.*, 1999). Furthermore, PPARγ ligands may also be valuable agents in cancer therapy. Indeed, it has been demonstrated that exposure to PPARγ ligands can induce terminal differentiation of human liposarcoma cells (Demetri *et al.*, 1999) and malignant breast epithelial cells *in vitro* (Suh *et al.*, 1999). However, treatment with PPARγ ligands can also have deleterious effects. Activation of PPARγ by oral TZD dosing has been linked to colon polyp formation in mice predisposed to intestinal neoplasia (LeFebvre *et al.*, 1998; Saez *et al.*, 1998). This would indicate that PPARγ activation via a high-fat diet could lead to an increased risk of colorectal cancer.

1.12.3 PPARδ

PPARδ (NR1C2) is the most ubiquitously expressed PPAR subtype (Xing *et al.*, 1995; Braissant *et al.*, 1996). Although its exact biological function remains elusive, studies by Gelman *et al.* (1999), and Leibowitz *et al.* (2000), have suggested that PPARδ may be involved in lipid metabolism, having a specific effect on high density lipoprotein levels without affecting plasma glucose or triglyceride levels. Administration of PPARδ agonists to insulin-resistant *db/db*[3] mice resulted in an induction of high density lipoprotein cholesterol levels (Leibowitz *et al.*, 2000). PPARδ has also been implicated in oligodendrocyte maturation and membrane sheet formation (Granneman *et al.*, 1998). Furthermore, PPARδ is the only subtype expressed in the uterus during embryo implantation in mice (Lim *et al.*, 1999). Expression of PPARδ mirrors the expression pattern of prostacyclin synthase, which produces prostacyclin PGI2, a cyclooxygenase-2

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[3] These mice have mutations in the *db* gene that encodes the leptin receptor. As a result, *db/db* mice are obese, hyperglycemic and hypertriglyceridemic.

(COX-2)-derived prostaglandin and a putative PPARδ ligand (Yu *et al.*, 1995 Lim *et al.* 1999). Since COX-2 null mice show defects in implantation and decidualization, it is hypothesised that COX-$2^{-/-}$ mice may be deficient in endogenous activators of PPARδ (Lim *et al.*, 1999). Recent evidence has also implicated PPARδ as a downstream effector of the tumour suppressor protein, APC, in colorectal cancer (He *et al.*, 1999). In common with other subtypes, PPARδ is activated by naturally occurring fatty acids such as ethyl esters of palmitic and oleic acids (Schmidt *et al.*, 1996), linoleic acid (Yu *et al.*, 1995), 8S-HETE (Krey *et al.*, 1997), as well as the synthetic ligands ETYA (Forman *et al.*, 1997), the arachidonic acid analog L-631,033 (Johnson *et al.*, 1997) and the fibrate derivative GW 2433 (Brown *et al.*, 1997).

1.13 Crosstalk Between Nuclear Hormone Receptors

A common theme in the regulation of metabolic processes is the convergence of different signaling pathways. Crosstalk between the various nuclear hormone receptor signaling pathways has been readily observed to mediate transcriptional regulation (Beato *et al.*, 1995). With respect to PPARs, several nuclear hormone receptors have been shown to bind to PPREs and differentially modulate PPAR function (Miyata *et al.*, 1993, 1996; Winrow *et al.*, 1994, 1998).

1.13.1 Retinoid X Receptor

Three RXR gene products (α, β, and γ) have been identified in mammals (Mangelsdorf *et al.*, 1990, 1992; Leid *et al.*, 1992) whose ligand is 9-*cis* retinoic acid (9-

cis RA). The RXRs are ubiquitously expressed, although individual RXR genes display unique but overlapping patterns of expression during development and in adult tissues (Levin *et al.*, 1992; Mangelsdorf, 1992; Liu and Linney, 1993; Dollé *et al.*, 1994). As promiscuous heterodimerization partners for various nuclear hormone receptors, RXRs participate in the expression of target genes involved in a wide range of biological processes. Two types of RXR heterodimeric complexes exist: non-permissive heterodimers such as RAR/RXR, TR/RXR, and VDR/RXR that can be activated only by the partner's ligand (Kurokawa *et al.*, 1994; Forman *et al.*, 1997), and permissive heterodimers like LXR/RXR and PPAR/RXR that can be activated by either partner's ligand (Kliewer *et al.*, 1992a; Willy *et al.*, 1995; Janowski *et al.*, 1996, Willy and Mangelsdorf, 1997). In the case of PPAR/RXR heterodimers, activation of the heterodimer complex can occur by each partner's individual ligand or when both receptors are liganded. The latter scenario results in an additive effect on transcriptional activation in transient transfections (Kliewer *et al.*, 1992a; Gearing *et al.*, 1993; Keller *et al.*, 1993). However, in a cellular context, the relative contributions of either the RXR or PPAR signaling pathways to PPAR/RXR-mediated gene transcription remains to be determined.

1.13.2 Thyroid Hormone Receptor α

Receptors for thyroid hormones exert a myriad of physiological effects by modulating the expression of genes involved in growth, development (Chatterjee, 1989) and lipid metabolism (Evans, 1988; Glass *et al.*, 1989; Chatterjee and Tata, 1992; Desvergne, 1994). Coincidently, these metabolic processes overlap those modulated by peroxisome proliferators. In fact, it has been demonstrated that the thyroid hormone, triiodothyronine (T3), attenuates peroxisome proliferator-mediated transcriptional induction of the genes encoding peroxisomal β-oxidation enzymes, suggesting crosstalk between these two signaling pathways (Takeda *et al.*, 1992; Pacot *et al.*, 1993; Yamada *et al.*, 1994). Evidence for a physiologically relevant connection between thyroid hormones and peroxisome proliferators is illustrated by the finding that PPAR activators such as fibrates promote adipocyte differentiation in a process that is similarly modulated by thyroid hormone (Gharbi-Chihi *et al.*, 1993). Studies by Bogazzi *et al.* (1994), Jow and Mukherjee (1995), Juge-Aubry *et al.* (1995), and Meier-Heusler *et al.* (1995), have further delineated that peroxisome proliferator and thyroid hormone signaling pathways converge at the level of their respective nuclear hormone receptors and that both PPARs and thyroid hormone receptors (TR) require heterodimerization with RXRα for DNA binding (Green and Wahli, 1994). In addition, PPARα can selectively down-regulate the transcriptional activity of TRs either by forming non-DNA-binding heterodimers with TRs (Bogazzi *et al.*, 1994; Jow and Mukherjee, 1995) or by competition for RXRα and other auxiliary proteins (Juge-Aubry *et al.*, 1995).

1.13.3 RevErbα

RevErbα (NR1D1) is an orphan member of the nuclear hormone receptor superfamily (Chawla and Lazar, 1993) that is encoded on the opposite strand of the TRα gene (Lazar *et al.*, 1989; Miyajima., 1989). RevErbα is expressed in a variety of tissue types, including adipocytes (Chawla and Lazar, 1993), skeletal muscle (Forman *et al.*, 1994), and liver (Forman *et al.*, 1994). Although the biological function of RevErbα remains elusive [illegible] has been implicated in adipogenesis (Chawla and Lazar, 1993), thyroid hormone signaling (Spanjaard *et al.*, 1994; Miyajima *et al.*, 1989; Lazar *et al.*, 1990) and muscle differentiation (Downes *et al.*, 1995). Recently, the gene for rat apolipoprotein A1, as well as the *CYP4A6* gene encoding a member of the cytochrome P450 fatty acid ω-hydroxylase family, have been shown to contain PPREs that bind, and are regulated by RevErbα (Hsu *et al.*, 1998; Vu-Dac *et al.*, 1998). Furthermore, RevErbα responds to the fibrate family of hypolipidemic drugs (Vu-Dac, N *et al.*, 1998). Human RevErbα has also been shown to mediate the transcriptional repression of its own promoter *in vitro* (Adelmant *et al.*, 1996). More recently, Gervois *et al.* (1999) have demonstrated that fibrate drugs induce the expression of the RevErbα gene through competition between RevErbα and PPARα/RXRα for binding to the autoregulatory RevDR2 site of the RevErbα gene.

Unlike many nuclear hormone receptors that bind to response elements as hetero- and homodimers, RevErbα is a member of a growing subclass of receptors that includes SF-1 (NR5A1) (Ikeda *et al.*, 1993), NGF-1B (NR4A1) (Milbrandt, 1988), RNR-1 (NR4A2) (Scearce *et al.*, 1993), RZRα (NR1F1) (Winrow *et al.*, 1998), and BD73 (NR1D2) (Retnakaran *et al.*, 1994), that bind notably as monomers (Adelmant *et al.*, 1996; Harding and Lazar, 1995). However, RevErbα can also bind as a homodimer to a RevErbα-DR2 element (Harding and Lazar, 1995). Moreover, unlike many nuclear hormone receptors, RevErbα lacks a conserved carboxyl-terminal activation domain (AF2) (Durand *et al.*, 1994; Harding and Lazar, 1995), which is suggested to be the cause of its repressive effects on transcriptional activation (Forman *et al.*, 1994; Harding and Lazar, 1995; Adelmant *et al.*, 1996; Zamir *et al.*, 1996). Monomeric RevErbα binds to the TGACCT/C consensus half-site flanked by a 5-base A/T-rich region, ACATT (Harding and Lazar, 1993; Forman et al, 1994; Harding and Lazar, 1995; Hsu *et al.*, 1998), in which the A at position +1 and the T at position +4 relative to the half-site are essential for high affinity binding (Harding and Lazar, 1993). This consensus half-site is found in both the AOx- and HD-PPREs.

1.13.4 Constitutive Androstane Receptor β

The nuclear hormone receptor constitutive androstane receptor β (CARβ) (NR1I4) (Choi *et al.*, 1997) heterodimerizes with RXRα to activate a subset of retinoic acid-response elements (Baes *et al.*, 1994; Choi *et al.*, 1997; Forman *et al.*, 1998) consisting of DRs related to the half-site consensus motif and separated by 2 or 5 base pairs. CARβ response elements include the retinoic acid receptor β2 response element (β-RARE) (Baes *et al.*, 1994; Choi *et al.*, 1997; Forman *et al.*, 1998) and the phenobarbital response element module (PBREM) of the *CYP2B* gene (Honkakoski *et al.*, 1998; Sueyoshi *et al.*, 1998). Interestingly, CARβ/RXRα possesses ligand-independent transactivity from both the β-RARE and PBREM in the absence of retinoids or any other exogenously added ligand (Choi *et al.*, 1997). CARβ is therefore among a growing number of orphan receptors, such as HNF-4 (NR2A1) (Sladek *et al.*, 1990; Ladias *et al.*, 1992; Carter *et al.*, 1993), steroidogenic factor 1 (SF-1) (Lynch *et al.*, 1993), nerve growth factor 1B (NGF-1B) (NR4A4) (Davis *et al.*, 1991; Wilson *et al.*, 1991; Paulsen *et al.*, 1992) and OR-1 (NR1H2) (Feltkamp *et al.*, 1999) that are capable of activating transcription in the absence of added ligand. Recently, Forman and coworkers showed that unlike the classical steroid/nuclear hormone receptors that are activated by their cognate ligands, CARβ binds the steroid androstane metabolites, androstanol and androstenol, which antagonize its ligand-independent transactivity (Forman *et al.*, 1998). This result proposes that CARβ could form part of a novel class of ligand-deactivated receptors (Picard, 1998).

1.13.5 Short Heterodimer Partner Receptor

The short heterodimer partner (SHP) (NR0B2) receptor is an unusual orphan nuclear receptor in that it lacks a typical DNA binding domain (Seol *et al.*, 1996). SHP was independently isolated by a yeast two-hybrid screen through its interaction with mouse CARβ (Seol *et al.*, 1996) and PPARα (Johansson *et al.*, 1999). Its 1.3 kb transcript is ubiquitously expressed in a variety of tissue types, with strong expression in the liver (Johansson *et al.*, 1999). In addition to the interaction between SHP and CARβ and PPARα in yeast, SHP has been shown to interact with a number of nuclear hormone receptors *in vitro*, including RAR, TR, RXR, ERs, and HNF-4 (Seol *et al.*, 1996; Masuda *et al.*, 1997; Johansson *et al.*, 1999; Lee *et al.*, 2000). In most cases, binding between receptors was enhanced in the presence of receptor-specific ligand (Seol *et al.*, 1996).

SHP has been proposed to act as a negative regulator of nuclear receptor transactivity, as demonstrated in transient transfections (Seol *et al.*, 1996, 1997; Johansson *et al.*, 1999). Because many nuclear hormone receptors are sensitive to SHP-mediated transcriptional repression, it is becoming increasingly obvious that the convergence of the SHP signaling pathway may be a critical regulatory mechanism in modulation of nuclear hormone receptor-mediated gene transcription.

1.14 Basal Transcription

A number of elegant studies have illustrated the complex mechanisms by which nuclear hormone receptors activate and repress transcription. It has long been thought that transcriptional activation by nuclear hormone receptors required either direct or indirect interaction with the basal transcription machinery (Baniahmed *et al.*, 1993; Tsai and O'Malley, 1994; Schulman *et al.*, 1995; Janknecht and Hunter, 1996; Glass *et al.*, 1997). The vast majority of eukaryotic genes are transcribed by RNA polymerase II, which produces a messenger RNA (mRNA) transcript of the target gene. A host of general transcription factors assemble at the core promoter in a highly ordered fashion, facilitating the recruitment of RNA polymerase II to initiate transcription (Zawel and Reinberg, 1995). These general transcription factors, which include transcription factors (TF) IID, TFIIB, TFIIE, TFIIF, and TFIIH, and the TATA binding protein (TBP), direct basal level transcription from promoters containing an initiation element called a TATA box (Goodrich *et al.*, 1996) located upstream (-25 to -30 bp) of the transcription start site. The assembly of the basal transcription factors begins with TFIID, which is a large complex consisting of at least nine subunits, TBP, and eight TBP-associated factors (TAFs) (Goodrich and Tjian, 1994). The TBP subunit binds specifically to the TATA sequence. Next, TFIIB is recruited to the complex followed by RNA polymerase II, which joins the DNA-bound transcription factors as a complex with TFIIF. TFIIE and TFIIH then assemble onto the complex, causing phosphorylation of RNA polymerase II, henceforth initiating transcription (Alberts *et al.*, 1994).

Gene expression is a precise and tightly regulated event that integrates various biological signals essential for normal growth and physiology. It is becoming increasingly apparent that transcription by RNA polymerase II promoters involves the action of both positive and negative regulatory factors (Goodrich *et al.*, 1996). Indeed, it has been demonstrated that a variety of transcriptional activating proteins such as coactivators (Section 1.15.1) and nuclear hormone receptors (*e.g.* RXR, THR, ER and PR) interact with various subunits of the general transcription machinery (Goodrich and Tjian 1994; Jacq *et al.* 1994; Schulman *et al.*, 1995; Schwerk *et al.*, 1995; Horwitz *et al.*, 1996) such as TBP and TAFs (Triezenberg 1995, Zawel and Reinberg, 1995).

1.15 Transcriptional Activation and Repression

A growing body of evidence suggests that nuclear hormone receptors form part of a large macromolecular complex of associated cofactors that acts as a protein "scaffold" to integrate signals between nuclear hormone receptors so as to either positively or negatively regulate transcription (Horwitz *et al.*, 1996; Nolte *et al.*, 1998; Torchia *et al.*, 1998). These cofactors are commonly referred to as coactivators and corepressors, respectively, and have been identified genetically and biochemically in both yeast and mammalian cells. A number of cofactors have been shown to possess intrinsic or associated enzymatic activities such as histone acetyl transferase, deacetylase, and protein methyl transferase activities (Chen *et al.*, 1999). These cofactors can exert their actions by remodelling chromatin, mediating interactions with auxiliary cofactors, introducing protein modifications, and promoting the assembly and/or stabilization of the transcription pre-initiation complex (Xu *et al.*, 1999). PPARs have been shown to interact with PPAR- or PPAR isoform-specific cofactors, as well as those shared amongst other nuclear hormone receptors (DiRenzo *et al.*, 1997; Dowell *et al.*, 1997, 1999; Meertens *et al.*, 1998; Miyata *et al.*, 1998; Nolte *et al.*, 1998; Treuter *et al.*, 1998) (Table 1-4)

1.15.1 Coactivators

Transcriptional activation by regulated transcription factors such as cAMP response element-binding protein (CREB) (Kwok *et al.*, 1994), mitogen-activated transcription factors (Arias *et al.*, 1994), as well as nuclear hormone receptors (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996) involves the recruitment of coactivator proteins to the nuclear hormone receptor in a ligand-dependent manner. There is an expanding list of identified coactivator proteins that include the CREB binding protein (CBP) and its homolog p300, CBP associated factor (p/CAF), steroid receptor coactivator 1 (SRC-1), the SRC-1 homolog transcriptional intermediary factor 2 (TIF2), activator of thyroid and retinoic acid receptor (ACTR), glucocorticoid receptor-interacting protein 1 (GRIP1), receptor interacting protein 140 (RIP140), PPARγ-binding protein (PBP), PPARγ-coactivator (PGC), receptor-associated coactivator 3 (RAC3), thyroid hormone receptor-activator molecule (TRAM), and peroxisome proliferator-activated receptor interacting protein (PRIP)/RAP250/ASC-2 (Lee *et al.*, 1999; Caira *et al.*, 2000; Zhu *et al.*,

Table 1-4. Nuclear Hormone Receptor Coactivators and Corepressors

Name	HAT/HDAC	Associated Factors*
ACTR	HAT	TR[h], RARβ[i]
AIB1	–	ER[h], RAR[e], VDR[j]
CARM-1	–	SRC-1[a], GRIP-1[a]
CBP/p300	HAT	AR[e], ER[e], PR[e], RAR[e], RXR[e], TR[e], VDR[e], SRC-1[a], p/CAF[b], TFIIB[b], TIF-2[h], p/CIP[h], TBP[n], PPAR[p]
DRIP/TRAP	–	VDR[b], TR[h]
GRIP-1/TIF-2	–	GR[e], CBP[h], ER[j], AR[j], PR[j], RAR[l], PPAR[m]
p/CAF	HAT	PR[b], SRC-1[b], TR[b], ACTR[h], CBP/p300[j], RAR[e], RXR[j]
p/CIP	HAT	CBP/p300[h]
PBP	–	PPARα[e], PPARγ[e], RAR[e], RXR[e], TRβ[h], VDR[e]
PGC-1	–	PPARγ[e], RAR[e], ER[e], SRC-1[a], CBP/p300[a]
PRIP	–	PPAR[q]
RAC3	HAT	RAR[n], VDR[o]
RIP140	–	PPARγ[e], RXR[e], TR[e], ER[b], PPARα[d], RAR[d], LXRα[d]
SMCC/ARC	–	VDR[b], TR[h]
SRC-1	HAT	PR[e], PPARα[e], PPARγ[e], PPARδ[e], TR[e], ER[b], GR[b], RXR[b], RAR[b], CBP/p300[b], p/CAF[h], HNF-4[h], TBP[n], TFIIB[e], VDR[e]
TRAM-1	–	TR[e]
TRIP-1/SUG-1	–	TR[b], RXR[b]
SMRT/TRAC-2	recruits HDAC	RAR[e], TR[e], RXR[c], PPARα[j], PPARγ[j], ER[j], PR[j], COUP-TFI[j], Sin3A[j], HDAC-1[j]
NCoR	recruits HDAC	RAR[c], TR[c], RXR[c], RevErb[f], PPAR[g], Sin3A[j], Sin3B[j]
Sin3	recruits HDAC	COUP-TF1[k], PR[k], NCoR[j], SMRT[j]
RDP3	HDAC	RAR[j]
TIF1α/β	recruits HDAC	RXR[h], RAR[h]

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[a] Leo and Chen, 2000
[b] Takeshita *et al.*, 1996
[l] Chen *et al.*, 1997
[m] Leers *et al.*, 1998
[q] Zhu *et al.*, 2000

[h] Shibata *et al.*, 1997b
[i] Zamir *et al.*, 1996
[j] Collingwood *et al.*, 1999
[n] Li *et al.*, 1997
[r] Gelman *et al.*, 1999

[c] Horwitz *et al.*, 1996
[g] Sande *et al.*, 1996
[k] Ogryzko *et al.*, 1998
[o] Chen *et al.*, 2000

[d] Miyata *et al.*, 1998
[e] McKenna *et al.*, 1999
[f] Voegel *et al.*, 1996
[p] Berger *et al.*, 1999

2000). The ability of coactivator proteins to potentiate ligand-dependent transactivation for several nuclear hormone receptors has been demonstrated *in vivo*.

The well-studied p160 family of coactivators is comprised of steroid receptor coactivator 1 (SRC-1)/NCoA-1, TIF2/GRIP1/NCoA-2, and p/CIP/ACTR/AIB1/ RAC3/TRAM-1, along with the CREB-binding protein (CBP) and the functionally related protein p300. These coactivators possess histone acetyltransferase activities and, as a result, are involved in the modification of chromatin (Kamei *et al.*, 1995; Oñate *et al.*, 1995; Bannister and Kouzarides, 1996; Hong *et al.*, 1996; Ogryzko *et al.*, 1996; Voegel *et al.*, 1996; Anzick *et al.*, 1997; Chen *et al.*, 1997; Li *et al.*, 1997; Spencer *et al.*, 1997; Takeshita *et al.*, 1997; Torchia *et al.*, 1997; Korzus *et al.*, 1998). Chromatin remodelling occurs through the acetylation of amino-terminal lysine-rich histone tails, which destabilizes histone-DNA interactions and causes the unwinding of DNA, making it more accessible to the transcriptional machinery (Chakravarti *et al.*, 1999). The p160 proteins are comprised of a number of structural motifs including three independent activation domains (AD-1, AD-2, and AD-3). In fact, the p160 family of coactivators anchor a complex comprised of the CBP/p300, p/CAF (Spencer *et al.*, 1997; Chen *et al.*, 1997), to nuclear hormone receptors through ligand-dependent AF2 interactions (Yao *et al.*, 1996; Anzick *et al.*, 1997). These so called general transcription coactivators enable full transcriptional activation by both nuclear hormone receptors and transcription factors such as STAT and NFκB. In essence, coactivators function primarily to recruit histone acetyl transferase activity to target promoters, allowing for the subsequent remodelling of chromatin (Freedman, 1999a and 1999b).

A second category of coactivators generally lacks histone acetyltransferase activity and are thought to act as "scaffolding" factors that link the receptor complex to the basal transcriptional machinery (Zhu *et al.*, 2000) either directly or via interactions with other auxiliary factors or multiprotein transcriptional complexes such as DRIP/TRAP and ARC/SMCC (Section 1.15.4). However, this distinction is not exclusive, since CBP and p300 have also been shown to interact with TFIIB (Kwok *et al.*, 1994) and TBP (Nakajima *et al.*, 1997), respectively. The large number of coactivator proteins in an active transcriptional complex may reflect the utility of distinct set(s) of coactivator proteins to bind specific nuclear hormone receptors to activate gene transcription from specific target genes in response to various physiological stimuli (Kawasaki *et al.*, 1998).

1.15.2 Structural Motif Requirements for Coactivator Interaction

The mechanism by which coactivators interact with nuclear hormone receptors is well established. The distinctive feature of coactivators is that they contain single or multiple LXXLL (where L is leucine and X is any amino acid) signature motifs (also called NR boxes) in a conserved central sequence used to directly interface with the AF2 domain of nuclear hormone receptors (McInerney *et al.*, 1998). Upon binding of ligand, α-helix H12 undergoes a conformational reorientation within the ligand binding domain structure, forming part of a "charged clamp" that interacts with the LXXLL motif of coactivators (Freedman, 1999a). Evidence for this mechanism is derived from experiments in which deletions or point mutations in the LXXLL residues abrogate coactivator-receptor interactions and abolish ligand-dependent transactivity (DiRenzo *et al.* 1997; Feng *et al.*, 1998; Meertens *et al.*, 1998; Nolte *et al.*, 1998). Interestingly, antagonists such as tamoxifen or raloxifene bound to estrogen receptor alter the position of the AF2 domain such that α-helix H12 occupies the hydrophobic cleft of the ligand binding domain, precluding the binding of coactivators (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). In addition to the integrity of the LXXLL motifs, binding between nuclear hormone receptors and coactivators is also influenced by sequences adjacent to the LXXLL motifs, spacing between LXXLL motifs, as well as residues around the periphery of the hydrophobic pocket (Darimont *et al.*, 1998). Furthermore, apart from interacting with nuclear hormone receptors, coactivator proteins also interact with each other to form a large macromolecular complex that synergistically potentiates transcription (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Yao *et al.* 1996; Freedman, 1999a). Examples include p/CAF which forms part of an approximately 20 subunit complex containing TAFs and TAF-like proteins (Ogryzko *et al.*, 1998), CPB/p300 and some of the p160 coactivators, as well as nuclear hormone receptors (Chen *et al.*, 1997; Blanco *et al.*, 1998). Interestingly, in some cases, these cofactor interactions involve the use of LXXLL motifs distinct from those used for interactions with nuclear hormone receptors. For example, SRC-1 contains specific carboxy terminal LXXLL signature motifs that are used for binding CBP/p300 (Oñate *et al.*, 1996; Torchia *et al.*, 1997; Dallas *et al.*, 1998; McInerney *et al.*, 1998).

1.15.3 Corepressors

Whereas coactivators facilitate transcriptional activation, transcriptional repression involves the recruitment of cofactors termed corepressors to nuclear hormone receptors in either the absence of ligand or in an antagonist-bound state (Chen and Evans, 1995; Jackson *et al.*, 1997; Perlmann and Evans, 1997; Shibata *et al.*, 1997b). In these states, nuclear hormone receptors assume a conformation distinct from agonist-bound receptors, with the surface of the H12 α-helix buried in the hydrophobic pocket, precluding it from interacting with coactivators, and thereby down-regulating transcription (Darimont *et al.*, 1998; Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). There are two classes of corepressors. The major class is formed by the silencing mediator of RAR and TR (SMRT) and the nuclear hormone receptor corepressor (NCoR) (Chen and Evans 1995; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995; Zamir *et al.*, 1997; Ordentlich *et al.*, 1999). These corepressors were initially identified as mediators of transcriptional silencing by RAR and TR in the absence of ligand (Baniahmad *et al.*, 1992, Chen and Evans, 1995; Kurokawa *et al.*, 1995; Nawaz *et al.*, 1995; Sande and Privalsky, 1996), but have since been shown to interact with a number of nuclear hormone receptors (Chen and Evans, 1995; Lee *et al.*, 1995; Zamir *et al.*, 1996, 1997; Heinzel *et al.*, 1997; Lavinsky *et al.*, 1998). The interactions between NCoR and SMRT with nuclear receptors are facilitated by one or more nuclear receptor interaction domains that contact the CoR box located between the hinge region and proximal ligand binding domain (Baniahmed *et al.*, 1992, 1995; Chen and Evans, 1995; Hörlein *et al.* 1995; Kurokawa *et al.*, 1995; Sande and Privalsky, 1996; Zamir *et al.*, 1996; Jackson *et al.*, 1997; Shibata *et al.*, 1997b). Mutations within the CoR box abolish ligand-independent repression (Shibata *et al.*, 1997b; Söderström *et al.*, 1997). However, for some nuclear hormone receptors, like ERs, RevErbα, and COUP-TFI which show no homology to the CoR box within any region of each receptor, suggests that other structural motifs may be involved in corepressor interactions (Downes *et al.*, 1996; Zamir *et al.*, 1996; Shibata *et al.*, 1997a). This may explain how NCoR and SMRT, which share sequence homology, have different preferences for specific nuclear receptors (Lavinsky *et al.*, 1998).

In vitro protein binding assays and co-immunoprecipitation studies have demonstrated that NCoR and SMRT are found in a multisubunit repressor complex with additional cofactors like Sin3 and RPD3 (Nagy *et al.*, 1997) whose interactions are mediated by specific repressor domains (RD) located on the corepressors (Horwitz *et al.*, 1996; Shibata *et al.*, 1997b). Sin3 and RDP3 subsequently recruit histone deacetylase activity through the histone deacetylase 1 (HDAC-1) protein (Alland *et al.*, 1997; Pazin and Kadonaga, 1997).

Transcription intermediary factor-1 (TIF-1) α and β form the minor class (LeDouarin *et al.*, 1995, 1996; vom Baur *et al.*, 1996) of corepressors that remain largely uncharacterized, although collectively it appears that corepressors form a multiprotein complex that functions to (1) recruit histone deacetylase activity to a target gene promoter to maintain chromatin in an inactive, hypoacetylated state, (2) prevent the functional nuclear hormone receptor-coactivator interaction, and (3) possibly block any transcriptional intracommunication between the AF1 and AF2 domains (Smith *et al.*, 1997)

1.15.4 Auxiliary Cofactors

A series of novel biochemical approaches has been used to isolate a large number of auxiliary factors forming a multisubunit complex that interacts with nuclear hormone receptors. These large complexes vary from 9 to 20 polypeptides and have relative molecular masses of 600 to 1000 kDa (Kingston, 1999). They have been referred to as vitamin D receptor-interacting proteins (DRIP) (Rachez *et al.*, 1998, 1999), thyroid hormone receptor-associated proteins (TRAP) (Fondell *et al.*, 1996), activator-recruited cofactors (ARC), and SRB/MED-containing cofactor complex (SMCC). These complexes are recruited to the AF2 domain in a ligand-dependent manner using an LXXLL motif from a single subunit of the complex (Rachez *et al.*, 1998). As an example, the DRIP205/TRAP220 subunits bind the nuclear hormone receptor and anchor approximately 13 to 15 proteins of a pre-formed DRIP/TRAP complex to the receptor. Sequence alignments have established that the DRIP and TRAP subunits are homologous, as are ARC and SMCC, while several DRIP/TRAP subunits show homology to those protein subunits of ARC/SMCC that associate with RNA polymerase II. (Gu *et al.*, 1999; Rachez *et al.*, 1999). This would suggest a general mechanism whereby these multi-subunit complexes function, in part, to recruit RNA polymerase II to various promoters. Furthermore, many of the protein subunits within each multi-subunit complex are also shared amongst the various complexes (Fondell *et al.*, 1996; Rachez *et al.*, 1998; Kingston, 1999; Näär *et al.*, 1999). For example, DRIP205, TRAP220 and ARC205 are identical, as are ARC100, DRIP100 and TRAP100. The importance of subunit sharing may emerge as another mechanism by which various signaling pathways are integrated for a desired transcriptional response (Näär *et al.*, 1999).

The emerging picture is that DRIP/TRAP and ARC/SMCC complexes represent very large protein complexes containing distinct and overlapping sets of protein subunits that interact with various transcriptional factors, cofactors, and RNA polymerase II to regulate different steps of transcription (Kingston, 1999).

1.15.5 Protein Methyltransferases

Methylation of DNA has long served as a central means of promoting transcriptional repression (Bird and Wolffe, 1999). In this mechanism, DNA methylation prevents the binding of basal transcription factors that require contact with cytosine residues in the major groove of DNA. Alternatively, methylation may also affect nucleosome stability, thereby restricting access of transcription factors to promoter DNA. However, it has been suggested that methylation may also serve to potentiate transcription through modification of histone proteins (Chen *et al.*, 1999). Chen and co-workers (Chen *et al.*, 1999) isolated a protein coactivator with arginine methyltransferase activity. This protein, termed CARM1 (coactivator-associated arginine methyltransferase 1), has been shown to bind the carboxyl-terminal region of the p160 coactivators, enhancing the transcriptional activity of nuclear hormone receptors such as the androgen receptor (AR) (NR3C4), ERs, and TRs.

In general, protein arginine methyltransferases catalyse the transfer of a methyl group from S-adenosylmethinoine to the guanidino group nitrogen atoms in arginine residues of specific proteins. In the case of CARM1, histone H3 has been shown to be methylated *in vitro* (Najbauer *et al.*, 1993; Gary *et al.*, 1998; Chen *et al.*, 1999). The presence of both protein methyl transferase and transcriptional coactivator activities in CARM1 are not mutually exclusive, as demonstrated by mutational analyses (Chen *et al.*, 1999), and suggests that methylation of histones and possibly other proteins plays a novel and important role in enhancing transcriptional activity.

1.16 General Mechanism of Transactivation and Transrepression

Figure 1-2 illustrates the dynamic relationship between transcriptional activation and repression. Most nuclear hormone receptors are constitutively nuclear and often bound to DNA in the absence of ligand (Chen and Evans, 1995; Horlein *et al.*, 1995; Horwitz *et al.*, 1996). In the unliganded state, nuclear hormone receptors form interactions with corepressor molecules that recruit histone deacetylase activity. This corepressor complex maintains chromatin in a repressive, transcriptionally inactive state (Chen and Li, 1998; Torchia *et al.*, 1998). Ligand binding induces a conformational change in the receptor, causing the dissociation of the corepressor complex but enhancing its affinity for a coactivator complex. The receptor-coactivator complex may contain one or more coactivator proteins that possess or recruit histone acetyl transferase activity, enabling the remodelling of chromatin from a repressed to an open, transcriptionally active state. This is followed by recruitment of an additional auxiliary multiprotein complex that ultimately forms positive interactions with the components of the general transcriptional machinery to initiates transcription.

1.17 Concluding Overview

The regulation of gene expression is a complex and dynamic process that involves the coordinated integration of various signaling pathways, cellular cofactors, and

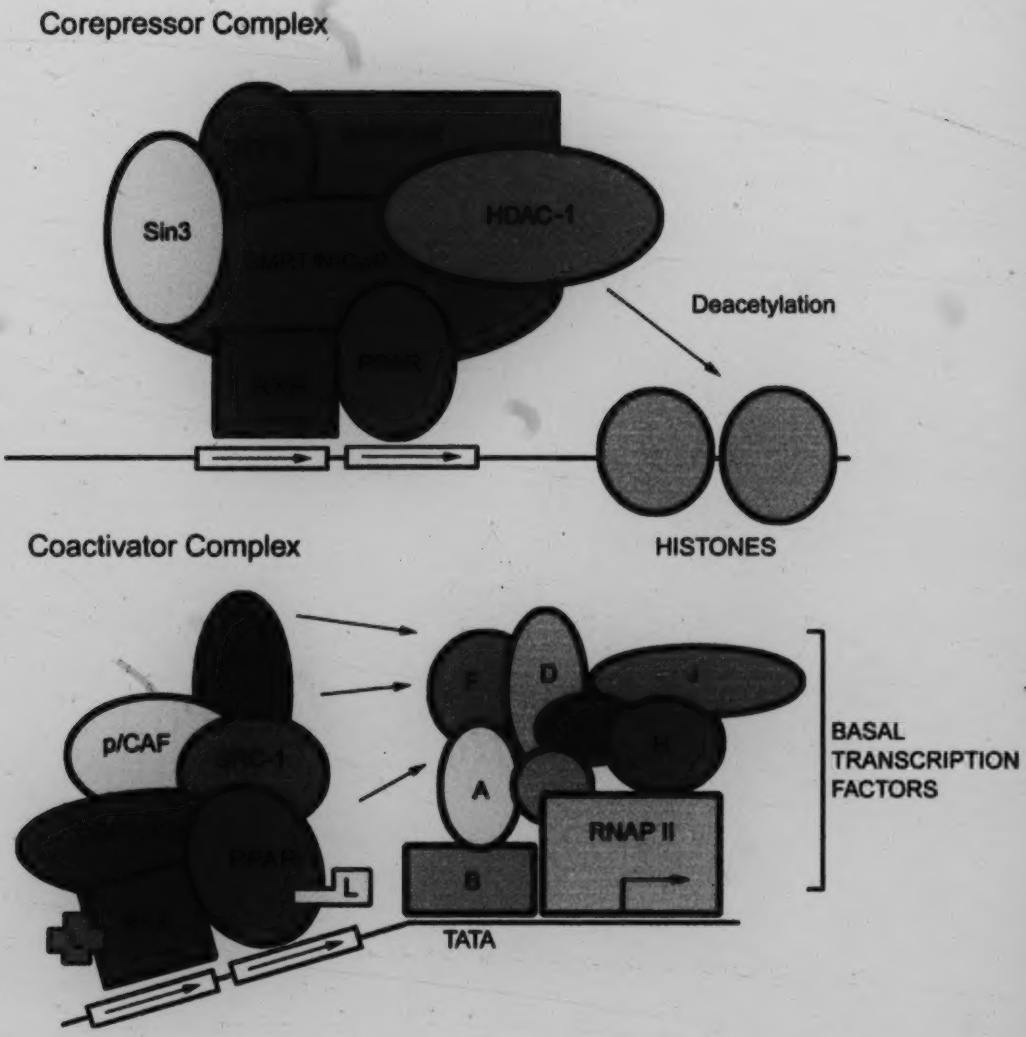
Corepressor Complex
Sin3
HDAC-1
SMRT/N-CoR
RXR
PPAR
Deacetylation
HISTONES
Coactivator Complex
p/CAF
SRC-1
PPAR
L
F
D
H
A
B
RNAP II
TATA
BASAL
TRANSCRIPTION
FACTORS

Figure 1-2 Model of Nuclear Receptor Corepressor and Coactivator Interactions

discreet genomic sequences. Nuclear hormone receptors constitute a distinct class of signaling molecules that link extra- and intracellular signals to transcriptional responses by binding to characteristic response elements in the promoter regions of target genes. The DNA target site generally consists of direct repeats of the TGACCT consensus half-site. Target site specificity is modulated, in part, by the core sequence, spacing and orientation of the half-sites, as well as by sequences flanking the TGACCT motif (Juge-Aubry *et al.*, 1997; Nishiyama *et al.*, 1998). DNA binding specificity is also subject to receptor promiscuity, such that different nuclear hormone receptors can bind to a given response element and, in a reciprocal manner, a specific receptor can bind to different response elements. Transcriptional regulation by these mechanisms can be seen with HNF-4 and COUP-TF homodimers and RZR monomers that bind to PPREs, displacing PPAR/RXR heterodimers from their natural binding site (Miyata *et al.*, 1993; Baes *et al.*, 1995; Winrow *et al.*, 1994, 1998). Additionally, response elements with a DR1 spacing are also recognized by RAR/RXR heterodimers and RXR homodimers (Nakshatri and Chambon, 1994; Fraser *et al.*, 1998). In comparison, PPARs can bind to both PPREs and RAREs. Therefore, in a cellular context, the relative amounts of each type of receptor plays an important role in the determining the final transcriptional response. The majority of nuclear hormone receptors bind to their target sites as heterodimers, although some receptors bind as homodimers or monomers. RXR is the typical heterodimerization partner for most nuclear hormone receptors including PPARs (Kliewer *et al.*, 1992b), RAR, TR (Zhang *et al.*, 1992) and VDR (Thompson *et al.*, 1998). Therefore, the availability of RXR can greatly influence the transactivation potential of nuclear hormone receptors (Chu *et al.*, 1995; Juge-Aubry *et al.*, 1995; Miyata *et al.*, 1996; DiRenzo *et al.*, 1997).

The molecular mechanism of nuclear hormone receptor activity is further complicated by the fact that receptor-interacting cofactors can modulate transcription. These include the p160 family of transcriptional coactivators and the transcriptional corepressor molecules of the NCoR/SMRT family, which either possess or recruit histone acetyl transferase or histone deacetylase activity, respectively. Furthermore, additional large multiprotein complexes termed DRIP/TRAP and ARC/SMCC can further potentiate nuclear hormone receptor transactivity through interactions with coactivator proteins and components of the basal transcriptional machinery, in a ligand-dependent manner. The availability of ligand and/or agonists critically influences the transcriptional potential of nuclear hormone receptors, either positively or negatively depending on the specific receptor. Therefore, it appears that transcriptional regulation by nuclear hormone receptors is a net transcriptional response generated, in part, by the availability of ligands and cellular cofactors, as well as by determinants within the DNA response elements. Together, they ensure the correct transcriptional response to both nutritional and metabolic cues.

1.18 Aims of This Thesis

Our laboratory has been interested in elucidating the molecular mechanisms by which PPARs regulate the transcription of genes involved in lipid metabolism. This thesis examines how members of the nuclear hormone receptor superfamily modulate PPAR-mediated gene transcription from the PPREs of the AOx and HD genes in response to peroxisome proliferators. More specifically, the binding properties of TRα, RevErbα, CARβ, and SHP have been investigated on both the AOx- and HD-PPREs *in vitro*. The biological effects of these receptors on PPAR/RXR-mediated transactivity have also been analysed *in vivo* using cultured mammalian cell lines. Analysis of the subtype- and response element-dependent differences in transactivity between PPARα and PPARγ from both PPREs was also carried out in the yeast *Saccharomyces cerevisiae*. The conclusions drawn from this body of work expand our understanding of the dynamic nature of PPAR-mediated gene transcription, provide further insight into the modulatory activities of nuclear hormone receptors, and establish a framework for continuing research on the various regulatory platforms that govern gene expression.

CHAPTER 2

Materials And Methods

2.1 Chemicals and Reagents

All reagents were of the highest quality available and, where required, were used according to the manufacturers' specifications, unless otherwise indicated.

acrylamide	SIGMA
acrylamide solution, ExplorER	J.T. Baker
acrylamide solution, Long Ranger	J.T. Baker
agar	Difco
agarose, electrophoresis grade	Gibco/BRL
L-amino acids	Sigma
ammonium persulphate	BDH
ampicillin	Sigma
antipain	Roche
aprotinin	Roche
benzamidine hydrochloride	Sigma
Bio-Rad protein assay dye reagent	Bio-Rad
bromophenol blue	BDH
charcoal	BDH
chymostatin	Sigma
Coenzyme A	Sigma
CSM-leu, ura, trp, his (complete supplements minus leucine, uracil, tryptophan, and histidine)	BIO 101
Coomassie Brilliant Blue R-250	ICN
Dextran T-70	Amersham-Pharmacia Biotech
DEPC (diethyl pyrocarbonate)	Sigma
DNA, from salmon testes, sodium salt	Sigma
DMF (dimethylformamide)	BDH
DTT (dithiothreitol)	ICN
EDTA (ethylenediaminetetraacetic acid)	Sigma
glass wool	Sigma
glutathione-Sepharose 4B	Amersham-Pharmacia Biotech
glycerol	BDH
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)	Roche
IPTG (isopropyl β-D-thiogalactopyranoside)	Vector Biosystems
leupeptin	Sigma
luciferin	Sigma
N,N'-methlenebisacrylamide	Gibco/BRL
nitrocellulose (Hybond-C)	Amersham-Pharmacia Biotech
NP-40 (Nonidet P-40)	BDH
o-nitrophenyl-β-D-galactosidase	Sigma

2-mercaptoethanol	BDH
MOPS (3-[N-morpholino] propanesulphonic acid	Sigma
Pefabloc SC	Roche
PEG-4000 (polyethylene glycol, average molecular weight 4,000)	Sigma
pepstatin A	Sigma
peptone	Difco
phenol, buffer-saturated	Gibco/BRL
PMSF (phenylmethylsulphonyl fluoride)	Roche
Ponceau S	Sigma
PPO (2,5-diphenyloxazole)	Sigma
RNasin	Promega
salmon sperm DNA (sonicated)	Sigma
Sephadex G-50	Amersham-Pharmacia Biotech
SDS (sodium dodecyl sulphate)	Sigma
sodium fluoride	Sigma
TEMED (*N,N,N',N'*-tetrametyhylethylene-diamine	Gibco/BRL
Tris (tris(hydroxymethyl)aminomethane)	Roche
tryptone	Difco
Tween 40 (polyoxyethylenesorbitan monopalmitate	Sigma
Wy-14,643	Wyeth Ayerst, Chemsyn Science Laboratories
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Vector Biosystems
yeast extract	Difco
YNB (yeast nitrogen base without amino acids)	Difco
15d-PGJ2	Cayman Chemical Company
9-*cis* retinoic acid	Sigma

2.2 Enzymes

2.2.1 DNA Modifying Enzymes

CIP (calf intestinal alkaline phosphatase)	NEB
DNA ligase, T4	Gibco/BRL, NEB, Roche,
DNA polymerase I, *Escherichia coli*, Klenow fragment	NEB
DNA polymerase, T4	NEB

restriction endonucleases	Gibco/BRL, NEB, Promega, Roche
polynucleotide kinase, T4	NEB
Taq polymerase (*Thermus aquaticus* DNA polymerase)	Perkin-Elmer, Roche

2.2.2 Other Enzymes

RNase A (ribonuclease A), bovine pancreas	Sigma, Roche

2.3 Multicomponent Systems

QIAprep MiniPrep Kit	Qiagen
Qiagen Plasmid Midi Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
Random Primers Labelling Kit	Roche
Ready-to-Go PCR Beads	Amersham-Pharmacia
Site-Directed Mutagenesis Kit	Roche
Sequenase Version 1.0 / 2.0 DNA Sequencing Kit	USB
TNT T7 /SP6 Coupled Reticulocyte Lysate System	Promega

2.4 Radiochemicals and Detection Systems

α-[^{32}P]dATP (3,000 Ci/mmol, 10 µCi/µL, Redivue)	Amersham-Pharmacia Biotech
L-[^{35}S]methionine (1,175 Ci/mmol, 10 mCi/mL)	ICN
enhanced chemiluminescence detection kits for immunoblotting and nucleic acid blotting, horseradish peroxidase-linked	Amersham-Pharmacia Biotech
nitrocellulose	Amersham-Pharmacia Biotech, Bio-Rad
X-ray film	Kodak

2.5 Molecular Size Standards

1 kb DNA ladder (75-12,216 bp)	Gibco/BRL
25 bp DNA ladder (25-500 bp)	Gibco/BRL
prestained markers for SDS-PAGE (6.5, 16.5, 25, 32.5, 47.5, 63, 83, 175 kDa)	NEB

2.6 Commercial Plasmid Vectors

2.6.1 E. coli vectors

pBluescript SKII (-/+)	Stratagene
pGEM7Zf(+)	Promega
pSG5	Invitrogen
pGEX-2T	Amersham-Pharmacia Biotech
pGEX-2TK	Amersham-Pharmacia Biotech
pGEX-4T	Amersham-Pharmacia Biotech
pRc/CMV	Invitrogen

2.6.2 Yeast Shuttle Vectors

pRS423*	ATCC
pRS424*	ATCC
pRS426*	ATCC
pRS313	Sikorski and Heiter, 1989
pRS314	Sikorski and Heiter, 1989

* (Christianson, *et al.*, 1992)

2.7 Antibodies

anti-guinea pig IgG (from goat), horseradish peroxidase-conjugated	Sigma
anti-rabbit IgG (from donkey), horseradish peroxidase-conjugated	Amersham-Pharmacia Biotech
anti-GST (polyclonal)	Clontech
anti-RXRα (polyclonal)	Marcus *et al.*, 1995
anti-TRα (monoclonal)	Santa Cruz Biotechnology

2.8 Oligodeoxyribonucleotides

Table 2-1 details the sequences and applications of oligodeoxyribonucleotides (oligonucleotides) used in this study. Oligonucleotides were synthesised at the DNA Sequencing Facility, Department of Biochemistry, University of Alberta, or using a Beckman Oligo 1000M synthesizer at the Department of Cell Biology, University of Alberta.

Table 2-1. Synthetic Oligodeoxyribonucleotides

Name	Sequence[a]	Application
HD-PPRE(x3)	5'-gatCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA 5'-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAG	Mammalian reporter plasmid
HD(M2)-PPRE(x3)	5'-gatCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA 5'-gatcTCAAATGTAGGTAATAGTTCAATATTTTAAAGGAGAG	Mammalian reporter plasmid
AOx-PPRE(x2)	5'-gatCCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTa 5'-gatctAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGGAAAG	Mammalian reporter plasmid
HD-PPRE	5'-gatCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA 5'-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAGG	Electromobility shift assay
M1-PPRE	5'-gatCCTATAATTTGACCTATTGAACTATTACCTACATTTGA 5'-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAATTATAGG	Electromobility shift assay
M2-PPRE	5'-gatCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA 5'-gatcTCAAATGTAGGTAATAGTTCAATATTTTAAAGGAGAGG	Electromobility shift assay
M3-PPRE	5'-gatCCTCTCCTTTGACCTATTGAAGTATTACCTACATTTGA 5'-gatcTCAAATATCTTCAATAGTTCAATAGGTCAAAGGAGAGG	Electromobility shift assay
M4-PPRE	5'-gatCCTCTCCTTTGACCTATTGAACTAATCTTCACATTTGA 5'-gatcTCAAATGTGAAGATTAGTTCAATAGGTCAAAGGAGAGG	Electromobility shift assay
AOx-PPRE	5'-gatCCTTTCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT 5'-gatAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGAAAGG	Electromobility shift assay
DR4	5'-gatcTTCTGACCTCCTGTGACCTGG 5'-gatCCAGGTCACAGGAGGTCAGAA	Electromobility shift assay
Nonspecific competitor	5'-GATCCCTACCCATACGACGTCCCAGACTACGCTTGAGCT 5'-AGCTCAAGCGTAGTCTGGGACGTCGTATGGGTAGGGATC	Electromobility shift assay
β-RARE(x2)	5'-cgcgtAAGGGTTCACCGAAAAGTTCACTCGCATAAGGGTTCA CCGAAAGTTCACTCGCATA 5'-gatctATGCGAGTGAACTTTCGGTGAACCCTTATGCGAGTGAA CTTTCGGTGAACCCTTA	Mammalian reporter plasmid
RXRα	5'-ATTACATCTAGACATGGACACCAAC 5'-ATTAGATCTGGTGGGCACAAAGGATG	PCR amplification of RXRα for cloning
PPAR-pBS	5'-CAAATCTCTGTTTTACGTAAAAATGGGTGAAACTC	Site-directed mutagenesis for expression in yeast
1AOxΔL1	5'-ccgggCCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTG 5'-tcgacAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGGAAAGGC	Yeast reporter plasmid
1HDΔL1	5'-ccgggCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGAG 5'-tcgacTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAGGC	Yeast reporter plasmid

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[a] Lowercase letters represent engineered restriction endonuclease sites

2.9 Commonly Used Buffered Solutions

Table 2-2 details the compositions of some commonly used buffered solutions.

Table 2-2. Commonly Used Buffers

Name	Composition	Reference
5 x KGB	0.5 M potassium glutamate, 125 mM Tris-acetate, pH 7.6, 50 mM magnesium acetate, 250 µg BSA/mL, 2.5 mM 2-mercaptoethanol	Hanish and McClelland, 1988
1x PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3	Ausubel *et al.*, 1996
10 x TBE	0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA	Maniatis *et al.*, 1982
1x TE	10 mM Tris-HCl, pH 7.0-8.0 (as required), 1 mM EDTA	Maniatis *et al.*, 1982
Buffer Z	60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$	Rose and Botstein, 1983
Breaking Buffer	20% (v/v) glycerol, 0.1 M Tris-HCl, pH 8.0, 1 mM DTT	Rose and Botstein, 1983
TBST	20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20	Huynh *et al.*, 1985
2x HBSS (pH 7.12)	280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4	Ausubel *et al.*, 1996
Lysis Buffer	25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM EDTA, 1% (w/v) Triton X-100, 10% (v/v) glycerol	Ausubel et al, 1996
2.5x Luciferase Assay Buffer	6.67 mM $MgSO_4$, 50 mM Tricine, 83.25 mM DTT, 0.25 mM EDTA 2.67 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_20$	Promega Technical Bulletin, 1990
1x PLATE	0.1 M Li-acetate, pH 7.5, 0.1 M Tris-HCl, pH 7.5, 50 mM EDTA, 40% (w/v) PEG 4000	Elble, 1992

2.10 Microorganisms and Culture Conditions

2.10.1 Bacterial Strains and Culture Conditions

The *Escherichia coli* strain DH5α (F^-φ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, $_{mK}^+$) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) (Gibco/BRL) was routinely used for amplification of plasmids (Section 2.11.1). *E. coli* BLR-DE3 (F^- *omp*T *hsd*SB(r_B^-, m_B^-) *gal dcm lon* (*srl-rec*A)*306::Tn10* (DE3)) (Novagen) was used for the production of glutathione-S-transferase (GST) chimeric proteins (Section 2.15.5). Bacteria were grown in a rotary shaker and, unless otherwise indicated, growth was at 37°C. Table 2-3 lists the bacterial culture medium employed in this study.

Table 2-3. Bacterial Culture Media

Medium	Composition	Reference
LB^{a,b}	1% tryptone, 0.5 % yeast extract, 1% NaCl, pH 7.5	Maniatis *et al.*, 1982

[a] Ampicillin was added to 100 µg/mL for plasmid selection, as required.

[b] For solid media, agar was added to 1.5%

2.10.2 Yeast Strains and Culture Conditions

Saccharomyces cerevisiae parental strain YPH500 (*Matα, ura3, lys2, ade2, trp1, his3, leu2*) was a gift from Drs. Sikorski and Heiter (Sikorski and Heiter, 1989), and used for expression of plasmid DNA. Yeast were grown in a rotary shaker at 30°C unless otherwise indicated.

2.11 Introduction of DNA into Microorganisms

2.11.1 Transformation of E. coli

In general, plasmids were amplified in transfection-competent, subcloning efficiency *E. coli* DH5α cells from Gibco/BRL and transformed according to the manufacturer's protocol. A 25 µL aliquot of cells was thawed on ice and mixed with 1 to 2 µL of a ligation reaction (Section 2.13.9) or 0.5 µL (approximately 0.5 µg) of plasmid

DNA. Cells were incubated on ice for 30 min, placed in a 37 °C water bath for 20 sec and immediately returned to ice for 2 min. One mL of LB was added to the cells, which were then shaken at 250 rpm for 45 min at 37 °C. Cells were spread onto LB-ampicillin plates and incubated at 37 °C overnight to allow colony formation. If required, 75 µL of 2% X-gal in dimethylformamide was spread on the surface of the agar plates before the cells were plated to allow for blue/white selection of colonies harbouring recombinant plasmids.

2.11.2 Electroporation

All electroporations were performed in BRL microelectroporation chambers (width approximately 0.15 cm) in a BRL Cell-Porator connected to a BRL Voltage Booster.

2.11.2-1 Electroporation of E. coli

High-efficiency transformation of *E. coli* with plasmids were carried out by electroporation using protease-deficient *E. coli* BLR(DE3). A 20 µL aliquot of cells was thawed on ice. Cells were gently mixed with 1 to 2 µL of plasmid DNA and transferred between the bosses of a prechilled microelectroporation chamber. Electroporation proceeded with a pulse of 395 V amplified to ~2.4 kV, using a capacitance of 2 µF and a resistance of 4 kΩ. Cells were then spread onto ampicillin-containing LB plates.

2.11.3 Transformation of Yeast by Li/Acetate/PEG

The yeast *S. cerevisiae* YPH500 was transformed with various effector plasmids and reporter genes (as indicated in the figure legends) by the lithium acetate procedure, essentially as described by the method of Elble (Ito *et al.*, 1983; Elble, 1992; Marcus *et al.*, 1995, 1996). A single colony of YPH 500 cells was used to inoculate 10 mL of YNBD (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with adenine (20 µg/mL), lysine (30 µg/mL), leucine (30 µg/mL), and tryptophan (30 µg/mL), as required. Cells were grown to an optical density of 1.0 at a wavelength of 600 nm using a Beckman DU 640 spectrophotometer. 500 µL of culture was transferred to a microfuge tube and subjected to centrifugation at 16 000 x *g* for 10 sec followed by decanting of the supernatant. 10 µL of single-stranded, high molecular weight salmon sperm DNA (10 µg/µL) (Sigma) (Section 2.11.4) and 1 µg of transforming DNA were added to the cells, followed by vortexing for 5 sec. A volume of 500 µL of PLATE buffer was added to the cell suspension, followed by brief vortexing. Samples were incubated overnight at room temperature and after centrifugation at 16 000 x *g* for 2 min, the supernatant was decanted and the cell pellet was resuspended in 100 µL of 1x TE. The cell suspension was spread onto YNBD-agar plates containing adenine (20 µg/mL), lysine (30 µg/mL), leucine (30 µg/mL), and tryptophan (30 µg/mL), as required. Plates were incubated for 3 days at 30°C, or until single colonies became visible.

2.11.4 Preparation of Single-Stranded High Molecular Weight Carrier DNA

One gram of DNA (type III, sodium salt, from salmon testes; Sigma D1626) was dissolved in 100 mL of TE by stirring overnight at 4 °C. The dissolved DNA was transferred to four 50 mL Falcon tubes and subjected to sonication using the Branson Sonifier Model 250 (Duty cycle 50%, Output power 40 %) twice for 30 sec. 1 µg aliquots of sonicated DNA were run on 0.8 % agarose gels (Section 2.13.7) to verify that DNA fragments were in the range of 2 kbp to 15 kbp in size with a mean size of approximately 7 kbp. After verification, an equal volume of buffer-saturated phenol was added to each tube and shaken vigorously on an orbital platform shaker for 20 min. Samples were subjected to centrifugation in a JS13.1 rotor at 13 000 x *g* for 10 min at 4 °C. The upper aqueous layer was removed, and the samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (26:25:1), followed by extraction with chloroform/isoamyl alcohol (25:1). DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol, thorough mixing by inversion and centrifugation at 13 000 x *g* for 20 min at 4 °C. Pellets were washed with ice-cold 70% ethanol and centrifuged at 13 000 x *g* for 10 min at 4 °C. Pellets were dried in a vacuum desiccator for 10 min and then were removed to a sterile beaker and incubated overnight at 4 °C in an appropriate amount of TE buffer (pH 8.0) to give a final concentration of 10 mg/mL. Dissolved DNA was boiled for 5 min and then immediately cooled in an ice-water bath. DNA concentration was determined from the absorbance at wavelength of 260 nm where 1 OD_{260} = 50 µg of DNA and then was divided into 1 mL aliquots that were stored at -20 °C.

2.12 DNA Isolation From Bacteria

2.12.1 Alkaline Lysis Preparation (Small Scale)

The alkaline lysis method of plasmid isolation was used essentially as described by Maniatis *et al.* (1982). A single bacterial colony was used to inoculate 2 to 3 mL of LB-ampicillin. Cells from 1.5 mL of a saturated culture were pelleted by microcentrifugation at 16 000 x *g* for 1 min. The supernatant was discarded, and the cell pellet was resuspended in 100 µL of 50 mM glucose, 25 mM Tris-HCL, pH 8.0, 10 mM EDTA. Cellular DNA was denatured by gentle mixing with 200 µL of 0.2 M NaOH, 1% SDS, followed by incubation on ice for a maximum of 5 min. Renaturation of plasmid DNA and precipitation of cellular proteins, high molecular mass RNA and chromosomal DNA were facilitated by the addition of 150 µL of potassium acetate solution (3 M K^+, 5 M acetate), followed by incubation on ice for 5 min. Cell debris was pelleted by microcentrifugation at 16 000 x *g* for 5 min at 4 °C . The supernatant was transferred to a clean 1.5 mL microfuge tube and subjected to extraction with an equal volume of phenol/chloroform/isoamyl alcohol (26:25:1), followed by extraction with an equal volume of chloroform/isoamyl alcohol (25:1). Aqueous and organic phases were separated by microcentrifugation at 16 000 x *g* for 2 min. DNA was precipitated by the addition of two volumes of absolute ethanol and microcentrifugation for 10 min at 16 000 x *g*. Excess absolute ethanol was discarded and the resultant DNA pellet was washed with 1 mL of 70% ethanol and dried in a rotary vacuum desiccator. The dried DNA pellet was dissolved in 50 µL of TE (pH 8.0) containing 20 µg RNase A/mL. Plasmids were analysed by restriction endonuclease digestion (Section 2.13.1) and, where necessary, by DNA sequencing (Section 2.14.1).

2.12.2 QIAprep MiniPrep Kit

Cells from 1.5 mL of a saturated LB-ampicillin culture were harvested by centrifugation for 2 min at 16 000 x *g*. Cell lysis and plasmid DNA isolation were carried out using the QIAprep MiniPrep Kit (Qiagen), according to the manufacturer's instructions.

2.12.3 Large Scale Plasmid Isolation

Qiagen DNA purification columns were used according to the manufacturer's directions for the isolation of plasmid DNA from large volumes of saturated culture. Cells from 500 mL of a saturated LB-ampicillin culture were harvested by centrifugation at 6 000 x *g* for 15 min at 4 °C. The supernatant was discarded, and the bacterial pellet was resuspended in 10 mL of P1 Buffer (50 mM Tris-HCL, pH 8.0, 10 mM EDTA, 100 μg RNase A/mL). 10 mL of P2 Buffer (200 mM NaOH, 1% SDS) was added with gentle mixing, followed by a 5 min incubation at room temperature, in order to denature cellular DNA. Protein and chromosomal DNA were precipitated by the addition of 10 mL of P3 Buffer (3.0 M potassium acetate, pH 5.5), gentle mixing and a 15 min incubation on ice. Precipitated products were separated from the plasmid DNA by centrifugation at 29 000 x *g* for 30 min at 4 °C . The supernatant was subjected to further clarification by centrifugation for 15 min at 29 000 x *g* at 4 °C and applied to a Qiagen Tip-500 ion-exchange column pre-equilibrated with 10 mL of QBT Buffer (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% (w/v) Triton X-100). The column was washed twice with 30 mL of QC Buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15 % isopropanol), and DNA was eluted with 15 mL of QF Buffer (1.25 NaCl, 50 mM Tris-HCl, pH 8.5, 15% isopropanol). DNA was precipitated by the addition of 10.5 mL of isopropanol and pelleted by centrifugation at 24 500 x *g* for 30 min at 4 °C. The DNA was washed with 20 mL of ethanol at room temperature, subjected to centrifugation at 24 500 x *g* for 5 min and air-dried at room temperature for 5 min. The dried DNA was resuspended in 500 mL water. The DNA concentration was measured by absorbance at a wavelength of 260 nm using a Beckman DU 640 spectrophotometer.

2.13 Standard DNA Manipulations

All procedures in this section were carried out essentially as described by Ausubel *et al.* (1999), except where noted.

2.13.1 Restriction Endonuclease Digestion

Restriction analysis of small-scale plasmid DNA preparations was used to verify the presence of a restriction fragment, determine the orientation of a fragment or to digest a plasmid vector for ligation purposes (Section 2.13.9). Typically, 1 μg of plasmid DNA was subjected to restriction endonuclease digestion according to the manufacturer's instructions

2.13.2 Dephosphorylation of 5' ends

In order to prevent the religation of plasmid vectors, 5'-terminal phosphate groups were removed using calf intestinal phosphatase (CIP). At the completion of a 10 μL restriction digest, 1 U of CIP was added, and the sample was incubated at 37 °C for 30 min. CIP was inactivated by heating the reaction at 75 °C for 15 min.

2.13.3 Phosphorylation of 5' ends

Phosphorylation of the 5'-termini of oligonucleotides was performed to enable their ligation into plasmid vectors or to other DNA fragments. A 30 μL reaction contained 20 μg of oligonucleotides, 3 μL of 10 x PNK buffer (700 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT), 3 μL of 10 mM ATP and 2 μL (10 U) of T4 polynucleotide kinase. The reaction was incubated at 37 °C for 2 hours and terminated by heating at 65 °C for 20 min.

2.13.4 Annealing Oligonucleotides

To generate double-stranded oligonucleotides for use in ligations (Section 2.13.9) or as probes for gel electromobility shift analysis (Section 2.14.5), complementary single-stranded oligonucleotides were annealed. A typical annealing reaction contained 10 pmol of each complementary oligonucleotide, 10 μL of 5 x Annealing Buffer (250 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$) and water to a final volume of 50 μL. Reactions were heated at 90 °C for 4 min in a heating block, which was removed from the heating element and allowed to cool gradually to room temperature. This facilitated the kinetics for proper annealing.

2.13.5 Creating Blunt-Ended Fragments

Restriction fragments that required the blunting of either 5' or 3' overhangs were subjected to treatment with either the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase, respectively. In the former, 5 U of Klenow and 100 μM of deoxyribonucleoside triphosphates (dNTPs) (*i.e.* 25 μM of each dNTP) were incubated in a final volume of 25 μL, and the reaction was incubated at 30 °C for 15 min. The reaction was terminated by subjecting the reaction to agarose gel electrophoresis (Section 2.13.7). Conversely, to blunt 3' overhangs, 2 μL (9 U) of T4 DNA polymerase and 100 μM dNTPs were added to a restriction digest reaction to a final volume of 25 μL (water added as necessary). The reaction was incubated at 11 °C for 20 min and terminated by heating at 75 °C for 15 min or by phenol/chloroform extraction (Section 2.13.6).

2.13.6 Phenol/Chloroform Extraction

Protein was removed from reactions containing nucleic acid by extraction with phenol/chloroform/isoamyl alcohol as described by Ausubel *et al.* (1998). Typically, an equal volume of phenol/chloroform/isoamyl alcohol (26:25:1) was added to the DNA solution, vortexed vigorously for 10 sec and subjected to microcentrifugation at 16 000 x *g* for 2 min at room temperature. Microcentrifugation resulted in phase separation, and the DNA-containing aqueous phase was transferred to a clean 1.5 mL microcentrifuge tube. The extraction was repeated with the an equal volume of chloroform/isoamyl alcohol (25:1). The aqueous phase was then transferred to a clean microcentrifuge tube and the plasmid DNA was precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ice-cold absolute ethanol. The sample was mixed by gentle inversion, incubated on ice for 15 min and subjected to microcentrifugation at 16 000 x *g* for 5 min at 4 °C. The supernatant was removed, and the pellet was rinsed with 1 mL of 70% ethanol and dried in a rotary vacuum desiccator. The dried pellet was dissolved in an appropriate amount of water or buffer, as required.

2.13.7 Agarose Gel Electrophoresis of DNA Fragments

To separate DNA fragments from enzymatic reactions, DNA samples were separated by agarose gel electrophoresis. A total of 0.2 volume of 6 x DNA sample dye (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) (Maniatis *et al.*, 1982) was added to an enzymatic reaction mixture, mixed by pipetting, and loaded into wells cast in agarose gels (0.8-1.5%, as required) in 1 x TBE containing 0.5 μg ethidium bromide/mL. Resolution of DNA fragments less than 400 bp in length was carried out using 3% gels consisting of 0.5% SeaKem GTG agarose and 2.5% NuSieve GTG agarose. Gels were run in 1 x TBE containing 0.5 μg of ethidium bromide/mL and subjected to electrophoresis at 10 V/cm. Fragments were visualized by placing the gel on an ultraviolet transilluminator (Photodyne, Model 3-3006) and photographed with Polaroid film (Polapan 667).

2.13.8 Purification of DNA Fragments

DNA fragments separated by agarose gel electrophoresis were isolated from the gel by excision with a sterile razor blade. The DNA was purified from the agarose gel either by electroelution or using *QIA*quick columns (Section 2.13.8).

2.13.8-1 Electroelution

The excised agarose gel slice containing the DNA fragment of interest was placed into the well of a unidirectional electroeluter (Model UEA, International Biotechnologies) filled with 0.5 x TBE. The bottom of the reservoir well was filled with 80 μL of high salt solution (7.5 M ammonium acetate, 0.25% bromophenol blue). Electroelution was carried out at 100 V for 30-60 min. A total volume of 350 μL containing the eluted DNA within the high salt solution and between the upper interface of 0.5 x TBE was transferred to a 1.5 mL microfuge tube. 25 μg of linear polyacrylamide (Gaillard and Strauss, 1990) and 1 mL of absolute ethanol (prechilled to -20 °C) were added, followed by gentle mixing by inversion. The DNA mixture was incubated at -80 °C for 15 min to allow the DNA to precipitate, and DNA was subsequently pelleted by microcentrifugation at 16 000 x *g* for 30 min at 4 °C. The pellet was washed with 1 mL of 70% ethanol and dried in a rotary vacuum desiccator. The DNA was dissolved in 20 μL of water.

2.13.8-2 QIAquick Columns

The QIAquick gel extraction kit (Qiagen) was used for the extraction of DNA fragments (100 bp to 10 kbp) from agarose gel slices or PCR reactions, according to the manufacturer's protocol. The QIAquick spin columns used in this kit use a uniquely designed silica-gel membrane that selectively adsorbs DNA in the presence of high concentrations of chaotropic salts in a pH environment of ≤7.5. DNA was eluted from the column by the addition of 30 to50 μL TE buffer (10 mM Tris-HCl, pH 8.5, 1mM EDTA).

2.13.9 Ligation of DNA Fragments

DNA fragments were prepared for ligation by enzymatic digestion. Ligation reactions were performed at a 5:1 molar ratio of insert to vector while maintaining total

DNA between 200 to 250 ng. Ligation reactions were carried out with 1 U of T4 DNA ligase in the buffer supplied by the manufacturer, ATP at a final concentration of 1 mM, and water to a final volume of 10 µL. Ligation reactions were incubated overnight at 16 °C, and ligation products were amplified by transformation of *E. coli*.

2.13.10 Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows for the amplification of DNA sequences *in vitro* through a succession of incubation steps at various temperatures (Mullis *et al.*, 1987). Additionally, the procedure can be used to introduce modifications into the amplified DNA sequences. Typically, double-stranded DNA is denatured by heat at 93 to 95 °C, and annealed to two primers that are complementary to the 3' ends of the DNA segment at a permissive, low temperature. The primers are then extended at an intermediate temperature, usually 72 °C. This cycle is repeated in order to amplify the DNA segment of interest. PCR makes use of a recombinant thermostable DNA polymerase (*Thermus aquaticus; Taq*) (Roche, Perkin Elmer) and 0.1 to 0.5 µg of DNA template, unless specified otherwise by the manufacturer's protocol. PCR was performed in a Robocycler 40 with a Hot Top attachment (Stratagene).

2.13.10-1 Taq Polymerase

Reactions using 2.5 U of *AmpliTaq* polymerase (Perkin Elmer) or 5 U of *Taq* polymerase (Roche) were carried out in a total volume of 100 µL containing the appropriate supplied buffer, 50 to 200 µM each of dATP, dCTP, dGTP and dTTP, and 0.2

to 1.0 μM of each specific oligonucleotide primer. The reaction mixture was overlaid with 50 μL of mineral oil (Sigma) to prevent evaporation, if needed. Upon completion of thermocycling, the PCR reaction was subjected to agarose gel electrophoresis.

2.13.10-2 Ready-To-Go PCR Beads

PCR reactions were carried out in a total volume of 25 μL containing less than 1 μg of template DNA, 25 pmol of each specific primer and one Ready-to-Go PCR bead (containing the necessary complement of buffer components, dNTPs, and *Taq* polymerase) (Amersham-Pharmacia Biotech). Upon completion of thermocycling, the PCR reaction was subjected to agarose gel electrophoresis.

2.13.11 PCR Product Purification

PCR reaction products that required purification without agarose gel electrophoresis were brought to a final volume of 100 μL with water, and the DNA was subsequently purified using QIAquick columns (Qiagen) according to the manufacturer's instructions. These columns use a special silica-gel membrane that adsorbs DNA (100 bp to 10 kbp), while excluding primers up to 40 nucleotides in length, in the presence of buffers containing high salt.

2.14 Analysis of DNA

2.14.1 DNA Sequencing

2.14.1-1 Template Preparation

Approximately 4 μg of double-stranded plasmid DNA was used as template for sequencing. The DNA was initially subjected to denaturation with alkali by the addition of 4 M NaOH, 4 mM EDTA, and water in a total volume of 28 μL. The mixture was incubated at room temperature for 5 min and then neutralized by the addition of 2.5 volumes of 2 M ammonium acetate. The DNA was precipitated with 2.5 volumes of absolute ethanol and incubation at -20 °C, followed by microcentrifugation at 16 000 x *g* at 4 °C for 30 min. The DNA pellet was washed with 70% ethanol and dried in a rotary vacuum desiccator.

2.14.1-2 Sequencing Reactions

Sequencing reactions were conducted using either Sequenase DNA Sequencing Kits (Versions 1.0 and 2.0; USB) or an automated ABI Genetic Analyzer, according to the manufacturer's protocol.

2.14.2 Sequenase Kits

This method generates sequences by the dideoxynucleotide chain-termination method of Sanger *et al.* (1977). DNA is sequenced using a genetic variant of the bacteriophage T7 DNA polymerase (Tabor and Richardson, 1990), which is devoid of any 3'→5' exonuclease activity found in the wild-type enzyme. The manufacturer's protocol was followed with the exception that 2 pmol of primer was used. Reaction products were run through denaturing acrylamide gels (5% Long Ranger or 5% ExplorER, in 0.6 x TBE or 1 x TBE buffer, respectively). Gels were dried at 80 °C for 45 min and subjected to autoradiography using Kodak X-AR film.

2.14.3 ABI Automated Sequencing

Sanger dideoxy sequencing (Sanger *et al.* 1977) reactions were conducted using the Stratagene Robocycler with attached Hot Top according to a standard protocol involving BigDye Terminator chemistry (PE Biosytems). AmpliTaq DNA polymerase FS is used in ABI PRISM cycle sequencing to elongate a DNA sequence by randomly incorporating dideoxy terminators, each tagged with a different fluorescent dye. Each dye emits fluorescence at a different wavelength when excited by an argon laser light, and therefore all four bases can be detected and distinguished by capillary electrophoresis using an ABI 310 Genetic Analyzer (PE Biosystems).

2.14.4 Radiolabelling of DNA Probes

To radiolabel oligodeoxyribonucleotides, complementary oligonucleotides were first annealed (Section 2.13.4) at a final concentration of 10 pmol/μL. Twenty pmols of double-stranded oligonucleotides were mixed with 5 μL of 5 x KGB buffer, 10 μL of [α-^{32}P]dATP (3000 Ci/mmol, 10 μCi/μL), 0.5 mM each of dGTP, dCTP, and dTTP, 1 μL (5U) of Klenow fragment, and water to a final volume of 25 μL. Samples were incubated at room temperature for 30 min. 75 μL of TE buffer was added to samples, and the radiolabeled probes were purified from unincorporated radionucleotide by centrifugation through Sephadex-G50 (Maniatis *et al.*, 1982; Section 2.14.4-1). Radionucleotide incorporation was measured by liquid scintillation counting in an LKB RackBeta 1209 scintillation counter. Radiolabeled probes were stored at -20 °C.

2.14.4-1 Spin Column Chromatography

A 1 mL syringe (BDH) was plugged with silanized glass wool (Sigma) and filled with Sephadex-G50 prepared in TE, pH 8.0. The syringes were placed into a 15 mL Falcon tube containing a cap-less microfuge tube lining the bottom of the Falcon tube. The spin column assemblies were placed in a clinical centrifuge and subjected to centrifugation for 4 min at 180 x *g*. Excess TE buffer was discarded, and the column was repacked with Sephadex G-50. This procedure was repeated until the column was filled with approximately 900 μL of packed Sephadex G-50. The column was then washed twice with 100 μL of TE buffer. The columns were immediately used for separating radiolabeled probes from unincorporated radionucleotide.

2.14.5 Gel Electromobility Shift Analysis

2.14.5-1 Binding Reactions with In Vitro Translated Proteins

In vitro translated proteins (as indicated in the figure legends) were incubated with 4 μg of BSA, 4 μg of poly dI•dC nonspecific competitor DNA, 4 μg of salmon sperm DNA, 4 μL of buffer C (20 mM HEPES, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). Water and unprogrammed lysate were added as appropriate to maintain a constant total reaction volume and a constant lysate concentration. Binding reactions were incubated at room temperature for 20 min and terminated by the addition of 1 to 2 μL of loading dye (30% glycerol (v/v), 0.5%

xylene cyanol, 0.5% bromophenol blue).

2.14.5-2 Binding Reactions with Yeast Extracts

To perform electrophoretic mobility shift analysis using yeast extracts, yeast transformed individually with plasmids pRS423:mPPARγ2, pRXR2μGPD, ymPPARα or the corresponding empty vectors, were grown to an OD_{600} of 1 in minimal media (YNBD supplemented with the appropriate amino acids) (Section 2.11.3). Extracts were prepared by glass bead disruption of cells in breakage buffer (20% (v/v) glycerol, 0.1 M Tris-HCl, pH 8.0, 1 mM dithiothreitol). Extracts containing 10 μg of total yeast protein were incubated with radiolabeled DNA probes, and protein/DNA complexes were then resolved by electrophoresis as described below.

2.14.5-3 Supershift Analysis

Protein binding reactions were carried out as above with the addition of 1 μL of monoclonal antibody specific for TRα or of polyclonal anti-RXRα antibody prior to the addition of labeled probe.

2.14.5-4 Electrophoresis

Binding reactions were analysed by electrophoresis at 4 °C on prerun 2.5 or 3.5% (as indicated in figure legends) polyacrylamide gels (30:1 acrylamide/*N,N'*-methylene bisacrylamide, 0.1% ammonium persulphate, 0.03% (v/v) TEMED), with 0.25 x Tris borate-EDTA (TBE) as running buffer, followed by autoradiography with Kodak X-ray film (BioMax). Where required, radiolabeled protein/DNA complexes were quantitated by phosphorimager analysis.

2.14.6 In Vitro Mutagenesis

Site-directed mutagenesis was carried out on single-stranded DNA templates using the Sculptor *In Vitro* Mutagenesis kit (Amersham-Pharmacia Biotech) according to the manufacturer's protocol.

2.15 Analysis of Proteins

2.15.1 Protein Determination

The protein concentration of a sample was measured by the method of Bradford (1976). An aliquot of a protein sample was brought to a volume of 100 μL with water, followed by the addition of 1 mL of Bio-Rad Protein Dye reagent. The sample was mixed by vortexing and incubated at room temperature for 10 min to allow for colour development. The absorbance of the sample was measured at 595 nm using a Beckman DU640 spectrophotometer, and protein concentration was determined by graphical analysis against the absorbance values of BSA protein standards of known concentration (1, 2, 5, 10 and 20 μg) dissolved in water.

2.15.2 Electrophoretic Separation of Proteins

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Protein samples were dissolved in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % sucrose, 10 mM DTT, 0.001% bromophenol blue, final concentration) and denatured by boiling for 5 min. Samples were subjected to discontinuous polyacrylamide gel electrophoresis. The stacking gel consisted of 3% acrylamide (30:0.8, acrylamide:*N,N'*-methylene-bisacrylamide), 60 mM Tris-HCl, pH 6.8, 0.1 % SDS, 0.1 % (v/v) TEMED, 0.1 % ammonium persulphate. Resolving gels were made of 10% acrylamide (30:0.8, acrylamide:*N,N'*-methylene-bisacrylamide), 370 mM Tris-HCl, pH 8.8, 0.1 % SDS, 0.1 % (v/v) TEMED, 0.042 % ammonium persulphate. Gels were run in the vertical dimension using a Bio-Rad Mini-Protean II vertical gel system at 50 to 200 V in SDS-PAGE running buffer (50 mM Tris-HCl, pH 8.8, 0.4 M glycine, 0.1 % SDS). Electrophoresis was allowed to proceed until the tracking dye reached the bottom of the gel.

2.15.3 Detection of Proteins

2.15.3-1 Coomassie Blue

Proteins separated by SDS-PAGE were visualized by staining the gels with 0.1% Coomassie Brilliant Blue R-250 dye in 10% (v/v) acetic acid, 35% (v/v) methanol for 30 min on an orbital shaker with gentle agitation. The gels were subsequently destained using 10% (v/v) acetic acid, 35% (v/v) methanol. The destain solution was replaced periodically until protein band resolution was distinct. Stained gels were then dried using a Bio-Rad gel dryer Model 583 for 1 hour at 80 °C.

2.15.3-2 Fluorography

Proteins labeled with ^{35}S-methionine were separated by SDS-PAGE as previously described (Section 2.15.2) and processed for fluorography by the method of Bonner and Laskey (1974). After electrophoresis, gels were placed directly in destain solution for 30 min with gentle agitation on an orbital shaker. The destain solution was discarded, and the gels were subjected to dehydration by two 30 min washes in 100% dimethylsulphoxide (DMSO). Gels were then incubated for 3 hours in 22.2% PPO (2,5-diphenyloxazole) dissolved in DMSO and subsequently rehydrated with two 15 min washes in water to precipitate the PPO. Gels were then dried at 60 °C, followed by autoradiography on Kodak X-ray film (BioMax or XK-1).

2.15.3-3 Immunoblotting

Electrophoretically separated proteins were transferred to nitrocellulose by electrophoresis using a BioRad western transblotter apparatus overnight at 100 mA in 20 mM Tris-HCl, pH 7.5, 150 mM glycine, 20% (v/v) methanol. Protein blots were stained in Ponceau S to verify the efficiency of transfer and subsequently destained in 1 x TBST (Table 2-2) containing 1% skim milk powder for 30 min at room temperature. Blots were washed three times with 1 x TBST for 5 min and subsequently incubated with a primary antibody solution in 1 x TBST containing 1% skim milk powder for 1 hour at room temperature with gentle agitation. The primary antibody solution was removed and blots were washed three times with 1 x TBST for 10 min and then incubated at room temperature for 30 min with a horsereadish peroxidase (HRP)-conjugated secondary antibody diluted in TBST containing 1% skim milk powder. After incubation, blots were washed three times for 10 min with 1 x TBST, and antigen-antibody reactions were detected by chemiluminescent exposure of Kodak X-ray film (XK-1).

2.15.4 In Vitro Transcription/Translation

2.15.4-1 Coupled Transcription/Translation

Proteins were synthesized *in vitro* using the TNT-coupled transcription/translation reticulocyte lysate system (Promega) according to the manufacturer's protocol. Typically, 0.5 to 1.0 μg of plasmid DNA was used in either 25 or 50 μL reaction volume with one of T7, T3, or SP6 RNA polymerase, as required.

2.15.4-2 Radiolabelling of Proteins

For synthesis of radiolabeled proteins, 10 μCi of [^{35}S]methionine (1175 Ci/mmol, 10 mCi/mL) was used in the coupled reaction.

2.15.5 GST Binding Assay

2.15.5-1 Preparation of Bacterial Lysates

A single colony of BL21 *E. coli* harbouring plasmids pGST-PPARα, pGST-RXRα, or pGST-SHP (Sections 2.18.8) was used to inoculate 3 mL of LB-ampicillin containing glucose at a final concentration of 2%, and the inoculum was incubated overnight at 37 °C. 500 μL of the saturated culture was used to inoculate 20 mL of LB/ampicillin/2% glucose for 3 hours at 37 °C. 20 μL of IPTG (100 mM) was added to the culture, and the culture was incubated for an additional 2 hours at room temperature. Cells were harvested by centrifugation at 16 000 x *g* for 10 min. Cell pellets were resuspended in 300 µL of 1 x PBS containing 50 mM EDTA, pH 7.5, and a protease inhibitor cocktail (leupeptin 1 µg/mL, pepstatin 1 µg/mL, aprotinin 1 µg/mL, chymostatin 0.1 µg/mL, antipain 2.5 µg/mL, 0.5 mM benzamidine hydrochloride, 5 mM NaF, 1 mM PMSF or 0.5 mM Pefabloc, 2 mM DTT). Cells were maintained on ice and then subjected to sonication on a Model 250 Branson Sonifier using a duty cycle of 50% and an output control setting of 3, three times for 10 sec each. Samples were subjected to microcentrifugation at 14 000 x *g* for 10 min at 4 °C, and the supernatant was transferred to a sterile 1.5 mL microfuge tube. The 1 x PBS/EDTA/protease inhibitor cocktail buffer containing 0.1 % (w/v) NP-40 was then added to a final volume of 500 µL. 100 µL aliquots were stored at -80 °C for later use.

2.15.5-2 Binding Reactions

Clarified lysates were thawed on ice and incubated with 2 or 4 µL of *in vitro* translated PPARα, RXRα, or SHP on an orbital rotator for 20 min at room temperature. To this a 20 µL GST-Sepharose 4B slurry was added, and the reaction was further incubated for 20 min on an orbital rotator. Binding reactions were then washed ten times with 800 µL of 1 x PBS/EDTA/0.1% NP-40/protease inhibitor cocktail by inverting the tube several times, followed by microcentrifugation at 14 000 x *g* for 10 sec. The supernatant was carefully discarded after each wash so as not to disturb the slurry/protein pellet. After washing, the slurry/pellet was resuspended in the approximately 20 µL of residual buffer remaining in the microfuge tube and 15 µL of 2 x SDS-PAGE sample buffer was added followed by denaturation by boiling for 5 min. Samples were subjected to centrifugation to pellet the GST-Sepharose 4B slurry, and the supernatant was subjected to electrophoresis via SDS-PAGE.

2.16 Transient Transfections

2.16.1 Cell Growth and Media

The BSC40 cell line used in this study was purchased from the American Type Culture Collection (CCL-26). These cells were chosen, because they contain low levels of endogenous PPARα and RXRα (Zhang *et al.*, 1992; Miyata *et al.*, 1993). Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) calf serum supplemented with 1% *L*-glutamine, 1% penicillin, and 1% streptomycin. Cells that were to be used in transient transfection were preincubated in DMEM containing dextran-coated charcoal-stripped fetal bovine serum (10%), supplemented with 4% *L*-glutamine, 1% penicillin, and 1% streptomycin. In addition, 0.1 M Wy-14,643 or an equal volume of vehicle (DMSO) was added as required.

2.16.2 Preparation of Dextran-Coated Charcoal-Stripped Fetal Calf Serum

12.5 g of charcoal (BDH) and 1.25 g of Dextran T-70(Amersham-Pharmacia Biotech), was added to 1M Tris-HCl, pH 7.5 at a final volume of 500 mL, and stirred overnight at 4 °C. For every 100 mL of fetal calf serum, 20 mL of dextran–coated charcoal (DCC) was pelleted by centrifugation at 4 300 x *g* for 10 min. The supernatant was discarded, and the pellet was mixed with fetal calf serum. Samples were shaken in a

56 °C waterbath for 30 min and then centrifuged at 4 300 x *g* for 15 min. Serum was stripped for a second time as before and was filtered through a 0.22 μM filter. DCC-FCS was stored in 50 mL aliquots at -20 °C.

2.16.3 Calcium Phosphate Method

Transient transfections were performed in BSC40 cells at 60 to 80% confluence using the calcium phosphate method of Graham and van der Eb (1973). Monolayer cultures in 10 cm dishes were routinely transfected with 5 μg of luciferase reporter plasmid DNA: pAOx(X2)*luc*, pHD(X3)*luc*, or pM2(X3)*luc*, as well as varying amounts of expression plasmid for PPARα, RXRα, TRα, RevErbα, CARβ or SHP, as indicated in the figure legends. In addition, effector plasmid dosage was kept constant by the addition of appropriate amounts of the corresponding empty vectors. Total DNA was normalized to 20 μg by the addition of sonicated salmon sperm DNA. Typically, a 2 mL solution was made of plasmid DNA, sonicated salmon sperm DNA, and 2 M $CaCl_2$. This solution was added to 2 mL of 2 x HEPES-buffered saline in a drop-wise fashion with continuous gentle vortexing. The mixed solution was allowed to incubate for 30 min at room temperature to allow for precipitation. A 960 μL aliquot of the solution was then added to cells preincubated in transfection media containing 100 mM Wy-14,643 (dissolved in DMSO) or 0.5% DMSO. After cells were incubated for 16 to 20 hours at 37 °C in the presence of 3% CO_2, the medium was replaced with fresh medium supplemented with Wy-14,643 or solvent, and the incubation was continued for an additional 16 to 20 hours at 37 °C and 7% CO_2. To harvest cells, the medium was removed, and cells were washed twice with

1 x PBS. 1 mL of ice-cold 1 x PBS was added to the plate, and the cells were scraped off using a Costar cell lifter (Model 3008, Corning), placed in a 1.5 mL Eppendorf tube, and microfuged briefly to pellet the cells. The pellets were then lysed by the addition of 100 μL of lysis buffer. Cell lysates were vortexed intermittently for 5 min and stored at -80 °C.

2.16.4 Luciferase Assay

Cell lysates prepared from transient transfections were thawed on ice and subjected to microcentrifugation at 14 000 x *g* for 10 min at 4 °C to pellet cell debris. The supernatant was assayed for luciferase activity using a luminometer (Bio-Orbit, Model 1253, Turku, Finland). An 80 μL aliquot of the cell lysate supernatant was added to 400 μL of luciferase reagent (0.5 mM ATP, 33.4 mM DTT, 0.5 mM luciferin, 0.27 mM Coenzyme A, 1 x luciferase assay buffer), briefly vortexed and placed in a luminometer cuvette.

2.17 β-Galactosidase Assay

Yeast transformants expressing PPARγ2, PPARα, and RXRα from low copy vectors (pRS313:mPPARγ2, ymPPARα, pRXRGPD314, respectively) or high copy vectors (pRS423:mPPARγ2, ymPPARα, and pRS424:RXRGPD, respectively) and harbouring the reporter genes 1AOxΔL1 or 1HDΔL1, were grown to an OD_{600} of 0.5 in YNBD supplemented with the appropriate amino acids. Cells were collected by low speed microcentrifugation and resuspended in YNBD containing 0.2% (v/v) Tween 40. Cultures were also supplemented with 10 μM 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2; Cayman

Chemicals, Ann Arbor, MI), 0.1 µM 9-*cis* retinoic acid, and/or vehicle (ethanol or DMSO, respectively). Cells were incubated for an additional 6 hours, harvested, and assayed for β-galactosidase activity as described by Rose and Botstein (1983), with the following modifications. Cells were resuspended in 100 µL of breaking buffer (20 % (v/v) glycerol, 0.1 M Tris-HCl, pH 8.0, 1 mM DTT) and disrupted by vortexing in the presence of acid-washed glass beads (Sigma). Lysates were diluted by the addition of 100 µL of breaking buffer. Assays were performed on 100 µL of diluted cell lysate brought to a final volume of 1 mL with 0.9 mL of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$). Samples were incubated at 28 °C for 5 min, at which time 200 µL of *o*-nitrophenyl-β-D-galactosidase (4 mg/mL) was added, and the incubation was continued for approximately 30 to 60 min to allow for colour development. The reaction was terminated by the addition of 0.5 mL of 1 M Na_2CO_3. β-galactosidase activity was measured at 420 nm using a Beckman DU 640 spectrophotometer, as described (Marcus *et al.*, 1995, 1996).

2.18 Plasmid Constructs

2.18.1 pCPSluc, pHD(X3)luc, and pAOx(X2)luc

The luciferase reporter plasmid pCPS*luc* is a luciferase expression vector containing the minimal promoter from the rat liver carbamoyl phosphate synthetase (CPS) gene (nucleotides -600 to -1 of the CPS promoter) (Howell *et al.*, 1989). The plasmid pHD(X3)*luc* contains three tandem copies of the HD-PPRE cloned into pCPS*luc*. It was constructed by inserting the oligonucleotide 5'-gatCCTCTCCTTTGACCTATTGAACT-

ATTACCTACATTTGA and its complement, 5'-gatcTCAAATGTAGGTAATAG-TTCAATAGGTCAAAGGAGAG (nucleotides -2956 to -2919 of the rat HD promoter), into the *Bam*HI site of pCPS*luc*.

pAOx(X2)*luc* contains two tandem copies of the rat AOx-PPRE generated by inserting the oligonucleotide 5'-gatCCTTTCCCGAACGTGACCTTTGTCCT-GGTCCCCTTTTGCTa and its complement, 5'-gatctAGCAAAAGGGGACCAGGAC-AAAGGTCACGTTCGGGAAAG (nucleotides -583 to -544 of the rat AOx promoter), into the *Bam*HI site of pCPS*luc*. Nucleotides designated in lower case were added to provide *Bam*HI/*Bgl*II ends.

2.18.2 prPPARα:Rc/CMV

Rat PPARα (rPPARα) cDNA was excised from pBluescript II SK(+) (a kind gift of Dr. Daniel Noonan, Ligand Pharmaceuticals, San Diego, CA) with *SpeI/EcoRV*, and the 2.6-kbp fragment was cloned into the *XbaI/EcoRI* sites of the expression vector pRc/CMV (Invitrogen, San Diego, CA).

2.18.3 phRXRα:SG5

To construct the expression vector harbouring human RXRα, the RXRα cDNA was excised from pSKXR3-1 (a kind gift of Dr. Ron Evans, Salk Institute, San Diego, CA) by digestion with *Eco*RI. The 1.8-kbp fragment was cloned directly into the *Eco*RI site of pSG5 (Stratagene).

2.18.4 pRSV-TRα

The mammalian expression vector for rat TRα (pRSV-TRα) was obtained from Dr. Vera Nikodem (National Institutes of Health, Bethesda, MD) and contains the rat TRα cDNA inserted as an *Hin*dIII/*Hpa*I fragment into pRSV (Bogazzi *et al.*, 1994). The reporter plasmid pTREpal*luc*, containing a high affinity palindromic TRE, was obtained from Dr. Chris Glass (University of California, San Diego, CA) (Glass *et al.*, 1988). The TRα *in vitro* expression vector was constructed by excising the full-length TRα cDNA from pRSV-TRα and cloning it into pGEM-7Zf(+) (Promega).

2.18.5 pRevErbα:SG5

The expression plasmid for human RevErbα (RevErbα:SG5) was constructed by excision of the human RevErbα cDNA as a *Bam*HI fragment from the plasmid pCMX:hRevErbα (a kind gift of Drs. Heather P. Harding and Mitchell A. Lazar, University of Pennsylvania, Philadelphia, PA) (Harding and Lazar,1993), followed by cloning of the fragment in the correct orientation into the *Bam*HI site of the plasmid pSG5 (Stratagene).

2.18.6 pCARβ:SG5

The CARβ:SG5 expression plasmid was constructed by excision of the cDNA for mouse CARβ as a *Sal*I/*Not*I fragment from the plasmid T7lacHisMyc/mCARβ (a kind gift from Dr. David D. Moore, Massachusetts General Hospital, Boston, MA) (Choi *et al.*, 1997), followed by its cloning as a blunt fragment into the *Sma*I site of pGEM-7Zf(+)

(Promega), removal as a n *Eco*RI/*Bam*HI fragment, and insertion into the EcoRI/BglII site of the eukaryotic expression vector pSG5. The integrity of the CARβ:SG5 construct was confirmed by sequencing.

2.18.7 pβRARE(X2)TKluc

The βRARE(X2)TK*luc* reporter plasmid was constructed by synthesizing oligonucleotides containing two copies of the β-RARE (bold) and overhangs for *MluI* and *Bgl*II (lowercase) 5'-cgcgtAAG**GGTTCA**CCGAAA**AGTTCA**CTCGCATAAG**GGTTCA**CCGAA**AGTTCA**CTCGCATA and its complement, 5'-gatctATGCGAG**TGAACT**TTCGG**TGAACC**CTTATGCGAG**TGAACT**TTCGG**TGAACC**CTTA. The oligonucleotides were phosphorylated with T4 polynucleotide kinase and ATP, annealed, and cloned into the pGL2 control vector (Promega) via *Mlu*I and *Bgl*II sites. The SV40 promoter was excised by digestion with *Bgl*II and *Hin*dIII and replaced with a 170 bp fragment of the TK promoter, which was excised from the plasmid TK-*luc*-pGL2 (Rachubinski *et al.*, 1999) by digestion with *Bgl*II and *Hin*dIII. The integrity of the construct was verified by sequencing.

2.18.8 pSHP:SG5

The plasmid pSHP:SG5 was obtained from Dr. Eckardt Treuter (Department of Biosciences at Novum, Karolinska Institute, Sweden) (Johansson *et al.*, 1999). pGST:PPARα was constructed by inserting the *Bam*HI fragment from pSG5-mPPARα (Tugwood *et al.*, 1992) into the *Bam*HI site of pGEX-2TK (Pharmacia) (Miyata *et al.*, 1998). pGST:RXRα was made by cloning the *Bgl*II fragment from pGEM7Zf:RXRα (see below) into the *Eco*RI site of pGEX-2T (Pharmacia) after first generating blunt ends with Klenow (Miyata *et al.*, 1998). pGST:SHP was constructed by ligating the *Eco*RI fragment from pSHP:SG5 into the *Eco*RI site of pGEX4T (Pharmacia) in the correct orientation.

2.18.9 ymPPARα and cmPPARα

Yeast high-copy 2μ vectors expressing mouse PPARα (mPPARα) was constructed by excision of the mPPARα cDNA from pPPAR/SG5 (Issemann and Green, 1990) as a 1.8-kbp *Bam*HI fragment. This fragment was cloned into the *Bgl*II site of the phosphoglycerate kinase (PGK) promoter/terminator, which had been inserted into the *Hin*dIII site of the yeast shuttle vector pRS426 (Christianson *et al.*, 1992). The entire mPPARα/PGK cassette was released as a *Bam*HI/*Xho*I fragment cloned into the vector pRS423 to generate ymPPARα and into the *CEN*-vector pRS313 to generate cmPPARα (Marcus *et al.*, 1995).

2.18.10 pRXR2μGPD, pRXRGPD314, and pRS424:RXRGPD

High copy and low copy expression plasmids for human RXRα under the control of the glyceraldehyde phosphate dehydrogenase promoter (GPD) was constructed as follows. The 1.8-kbp fragment of the RXRα cDNA was amplified from pSKXR3-1 using the forward primer 5'-ATTACATCTAGACATGGACACCAAC and the reverse primer 5'-ATTAGATCTGGTGGGCACAAAGGATG, treated with Klenow fragment, and cloned into the SmaI site of the pGEM7Zf(+) vector. A 1.4-kbp fragment was excised with *Bgl*II and cloned into the *Bam*HI site of p2μGPD (*URA3*, 2μ) (a kind gift from S. Lindquist, University of Chicago, Chicago, IL) to generate pRXR2μGPD. The 2.1-kbp RXR:GPD cassette was removed by digestion with *Xho*I and *Spe*I and ligated into the pRS314 and pRS424 to yield the low copy plasmid pRXRGPD314 and the high copy plasmid pRS424:RXRGPD, respectively.

2.18.11 pRS423:mPPARγ2 and pRS313:mPPARγ2

To construct expression vectors for mouse PPARγ2 (mPPARγ2), a 1.8-kbp fragment containing the mPPARγ2 cDNA was excised from the PPARγ2/SPORT plasmid (a kind gift of Dr. Bruce Spiegleman, DanaFarber Cancer Institute, Boston, MA) (Tontonoz *et al.*, 1994) with *Spe*I/*Mlu*I, repaired with the Klenow fragment and cloned into the *Sma*I site of pBluescript II SK(-) (Stratagene). Site-directed mutagenesis of the region around the translation initiation codon with the oligonucleotide 5'-CAAATCTCTGTTTTACGTA**AAAATG**GGTGAAACTC was performed to create a unique *Sna*BI site (underlined) and to alter the initiation codon context (bold) for efficient translation in yeast. The 1.8-kbp fragment was excised with *Sna*BI and *Xho*I (thereby removing the 5' untranslated region of the cDNA), made blunt with Klenow fragment, and cloned into the *Bam*HI site of the plasmid pUGPD (*URA3*, *CEN6*, *ARSH4*) (kindly provided by Dr. Susan Lindquist, University of Chicago, Chicago, IL). The GPD:mPPARγ2 cassette was liberated with *Spe*I/*Xho*I and cloned into pRS423 (*HIS3*, 2μ) (Christianson *et al.*, 1992) and pRS313 (*HIS3*, *CEN6*, *ARSH4*) (Sikorski and Heiter, 1989) to generate the plasmids pRS423:mPPARγ2 and pRS313:mPPARγ2, respectively.

2.18.12 1AOxΔL1 and 1HDΔL1

Single copies of the AOx-PPRE (bold) double-stranded oligonucleotide 5'-CCTTTCCCGAACG**TGACCTTTGTCCT**GGTCCCCTTTTGCT or of the HD-PPRE double-stranded oligonucleotide 5'-CC**TCTCCTTTGACCTATTGAACTATTACCT**-ACATTTGA, were excised as *Xma*I/*Sal*I fragments from pSP73 (Marcus *et al.*, 1995) and cloned into the pΔL1 (ura +) *lacZ* vector (Marcus *et al.*, 1995) to make the *lacZ* reporter plasmids 1AOxΔL1[4] and 1HDΔL1[5], respectively.

2.18.13 pAOx(X2)GL and pHD(X2)GL

Plasmids containing the HD- and AOx-PPREs upstream of the minimal SV40 promoter were constructed by cloning the corresponding synthetic oligonucleotides into the *Bam*HI site of the enhancerless SV40 promoter/luciferase expression plasmid pGL2 (Promega, Madison, WI). The resultant plasmids, pAOx(X2)GL[6] and pHD(X2)GL[7], contain two tandem copies of the respective PPRE in the forward orientation.

[4, 5] Constructed by Sandra L. Marcus, University of Alberta, Edmonton, Canada

[6, 7] Constructed by John Hunter, McMaster University, Hamilton, Canada

CHAPTER 3

Crosstalk Between the Thyroid Hormone and Peroxisome Proliferator-Activated Receptors in Regulating Peroxisome Proliferator-Responsive Genes[§]

[§] A version of this chapter has been published. Hunter, J., Kassam, A., Winrow, C. J., Rachubinski, R. A., and Capone, J. P. *Mol. Cell. Endocrinol.* 116:213-221 (1996) and Winrow, C. J., Kassam, A., Miyata, K.S., Hunter, J., Capone, J. P., and Rachubinski, R. A. *Ann. N. Y. Acad. Sci.* 804:214-230 (1996). Used with permission from Elsevier Science and the New York Academy of Sciences, respectively.

3.1 Abstract

Peroxisome proliferators and thyroid hormones have overlapping metabolic effects and regulate a subset of genes involved in maintaining lipid homeostasis. Thyroid hormone receptors mediate cellular responses which, like PPARs, affect the expression of many genes that are important for lipid metabolism, cellular growth, and development (Hertz *et al.*, 1993). The effects of thyroid hormone receptor α (TRα) on DNA binding *in vitro* and transcriptional activation *in vivo* by rat peroxisome proliferator-activated receptor α (PPARα) was examined. Gel mobility shift analysis demonstrated that TRα was capable of binding on its own and cooperatively with the 9-*cis* retinoic acid receptor α (RXRα) to the rat acyl-CoA oxidase (AOx) PPRE and of inhibiting the binding of PPARα/RXRα heterodimers on this element. This inhibition was the result of competition between TRα and PPARα for limiting amounts of the heterodimerization partner RXRα and for binding to the PPRE *in vitro*. Interestingly, cotransfection of a TRα expression plasmid into mammalian cells resulted in the potentiation of the peroxisome proliferator Wy-14,643- and PPARα/RXRα-dependent transcriptional induction of a reporter gene containing the AOx-PPRE. TRα therefore appears to cooperate with RXRα and PPARα to positively modulate peroxisome proliferator-dependent transactivation *in vivo*. These findings suggest that there is crosstalk between the thyroid hormone and peroxisome proliferator signaling pathways in the regulation of peroxisome proliferator-responsive genes.

3.2 Results

3.2.1 TRα and TRα/RXRα heterodimers bind to the AOx-PPRE

TRα/RXRα heterodimers bind with high affinity to TGACCT direct repeats spaced by four nucleotides (DR4) (Umesono and Evans, 1989; Umesono *et al.*, 1991), whereas PPARα/RXRα heterodimers bind preferentially to direct repeat response elements configured as DR1, as found in both the AOx- and HD-PPREs (Tugwood *et al.*, 1992; Zhang *et al.*, 1993). However, natural thyroid hormone response elements (TRE) are configured in various manners and orientation and can be recognized by TR monomers, homodimers, or heterodimers with RXRα and other partners (Desvergne, 1994). To explore whether TRα recognizes the AOx-PPRE and/or modulates PPARα/RXRα protein/DNA interactions on this element, we carried out DNA binding assays with *in vitro* transcribed and translated receptors. TRα /RXRα heterodimers could bind cooperatively to a double-stranded oligonucleotide containing a DR4 element (Fig. 3-1, lane d). TRα could also bind to the DR4 element weakly as a monomer (lanes b, d, e and g). PPARα alone or with RXRα or TRα did not form a complex on the DR4 element. Mobility shift experiments carried out with the AOx-PPRE are shown in Figure 3-2. As previously demonstrated (Marcus *et al.*, 1993), PPARα and RXRα did not bind the AOx-PPRE individually but did bind cooperatively (lane e). Interestingly, TRα alone formed a monomeric complex on the AOx-PPRE (lane c). However, coincubation of TRα and PPARα failed to form a complex on the AOx-PPREs (lane g). Conversely, in the presence of RXRα, TRα formed an additional complex on the AOx-PPRE that had a slightly faster electrophoretic mobility than that of the PPARα/RXRα complex (lane f).

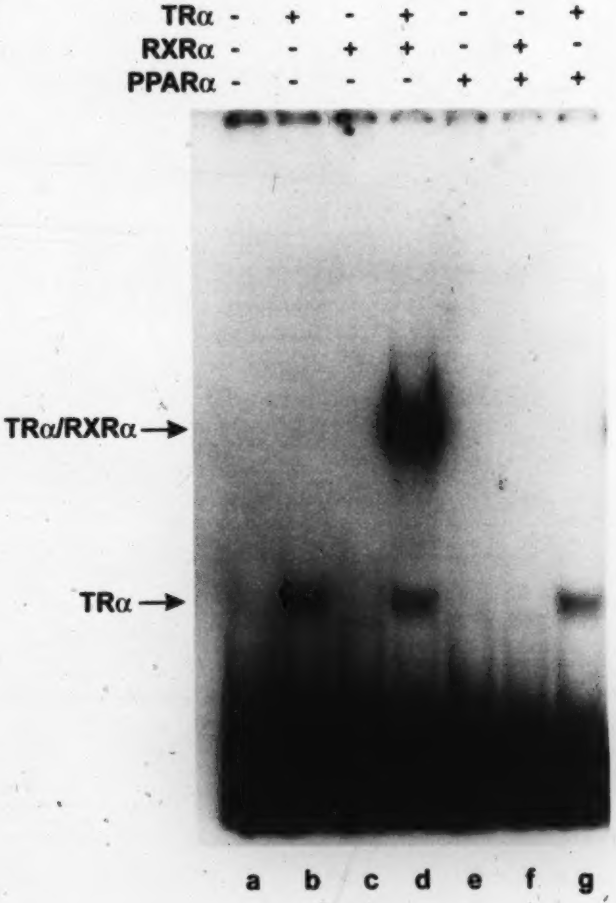


Figure 3-1. Interaction of TRα with a synthetic DR4 element. *In vitro* synthesized TRα, PPARα, and RXRα (1μL each) were used individually or in pairwise combinations (as indicated at top) in gel retardation assays with a radiolabeled synthetic DR4 probe. Reactions were normalized with unprogrammed lysate as appropriate. The positions of the TRα monomeric complex and the TRα/RXRα heterodimeric complex are indicated. Lane a is a control in which the DR4 probe was incubated with unprogrammed lysate.

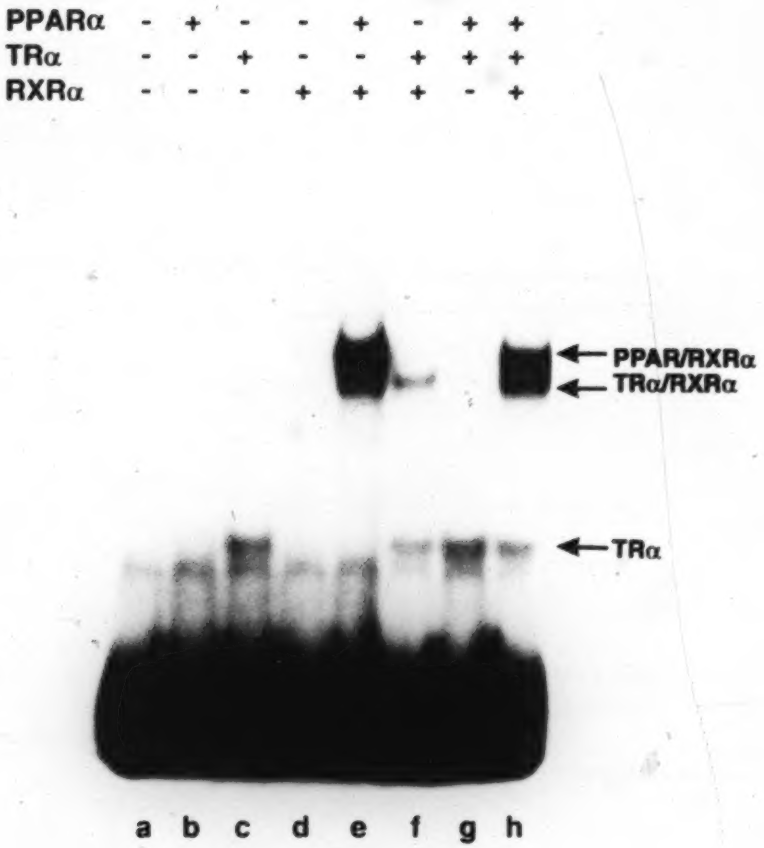


Figure 3-2. TRα binds to the AOx-PPRE on its own and in association with RXRα. *In vitro* synthesized receptors (1μL each) were incubated individually or in various combinations (as indicated at top) with radiolabeled AOx-PPRE probe and analyzed by gel retardation as in Figure 1. Reactions were normalized with unprogrammed lysate as appropriate. The positions of the monomeric TRα complex and the heterodimeric TRα/RXRα complexes are indicated. The TRα/RXRα complex has a slightly faster electrophoretic mobility than that of the PPARα/RXRα complex.

Coincubation of equal amounts of PPARα, RXRα, and TRα led to the formation of three distinct complexes, corresponding to PPARα/RXRα heterodimer and TRα/RXRα heterodimer, and TRα monomer complex (lane h). Under these conditions, the TRα/RXRα and PPARα/RXRα complexes formed with approximately the same efficiency, indicating that TRα and PPARα compete for limiting amounts of RXRα.

To determine if TRα was a component of these protein/DNA complexes, supershift analysis with antibodies to TRα and RXRα were carried out. Addition of anti-TRα antibodies to the binding reaction disrupted the formation of both the faster migrating TRα-dependent complex and the more slowly migrating TRα/RXR-dependent complex (Fig. 3-3, lanes d and h). In contrast, anti-RXRα antibodies disrupted the formation of only the more slowly migrating complex (lane g). Preimmune serum had no effect on complex formation (lane f). These results show that TRα was present in both the faster and more slowly migrating TRα-dependent complexes, whereas RXRα was only a component of the more slowly migrating complex.

The TRα/RXRα-dependent complex was sequence-specific, since it could be competed with unlabeled AOx-PPRE probe DNA but not with a nonspecific competitor oligonucleotide (Fig. 3-4, lanes i-k and e-g, respectively). TRα monomeric complex was similarly competed for by specific, but not nonspecific, competitor DNA; however, this competition required a 100- to 200-fold molar excess of competitor DNA compared to the 10- to 50-fold molar excess that was sufficient to disrupt the PPARα/RXRα and TRα/RXRα complexes. These results suggest that the TRα monomeric complex is more stable than the TRα/RXRα complex.

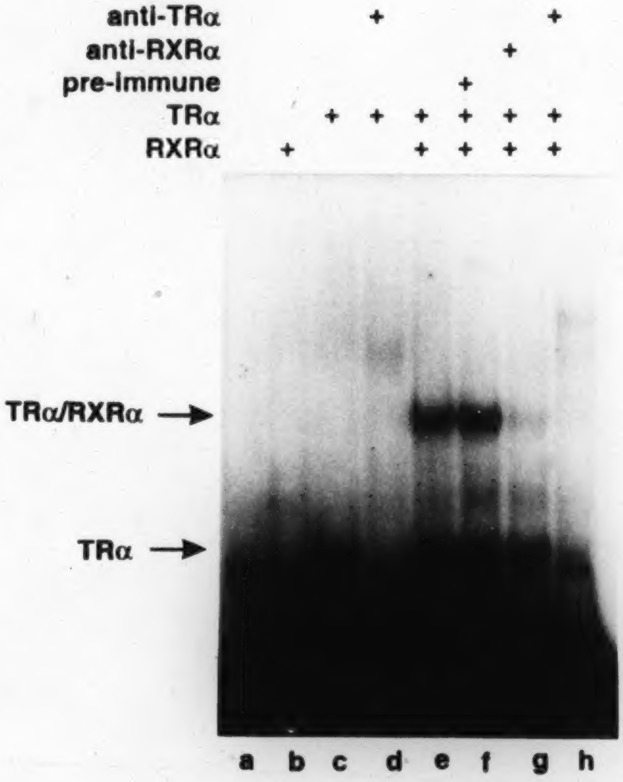


Figure 3-3. TRα is present in complexes formed on the AOx-PPRE. Gel retardation assays using radiolabeled AOx-PPRE were carried out with *in vitro* synthesized receptors (1 μL each) in the presence of preimmune serum (lane f), monoclonal anti-TRα antibodies (lanes d and h), or polyclonal anti-RXRα antibodies (lane g), as indicated. One μL of preimmune serum or antibodies was added along with the indicated receptors prior to the addition of the AOx-PPRE probe. Reactions were normalized with unprogrammed lysate as appropriate. Inclusion of anti-TRα antibodies resulted in the loss of both the fast migrating and slowly migrating complexes (lanes d and h), while inclusion of anti-RXRα antibodies resulted in the loss of only the slowly migrating complex (lane g). Specific supershifted complexes produced by addition of anti-TRα and anti-RXRα antibodies were apparent after longer exposure. Lane a is a control in which the AOx-PPRE was incubated with unprogrammed lysate.

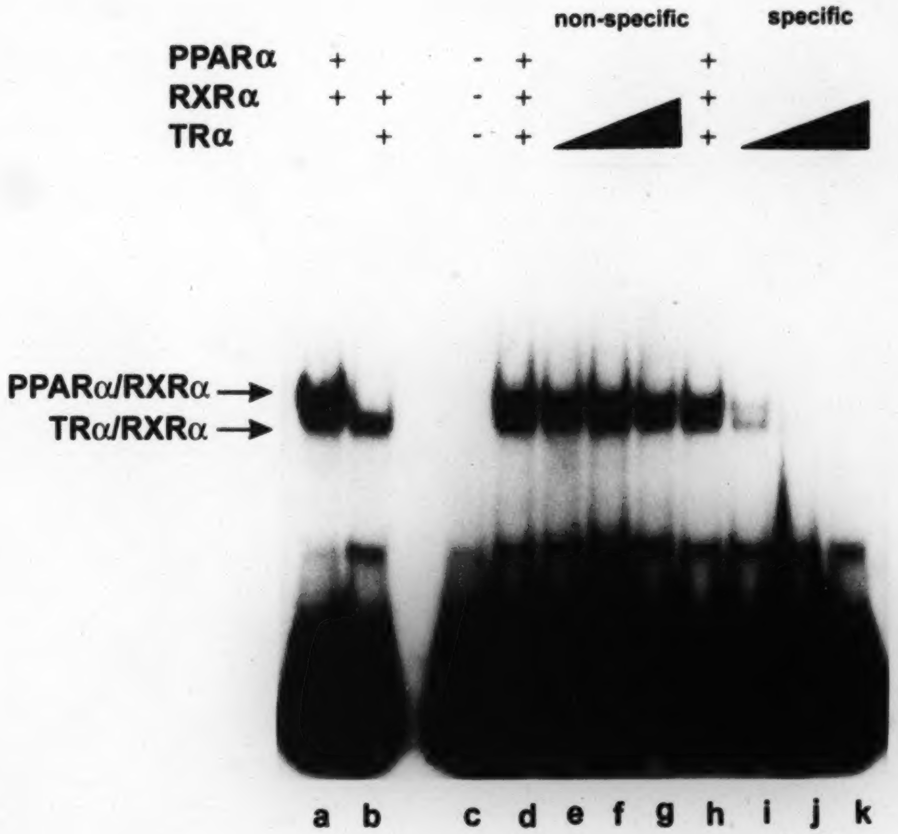


Figure 3-4. TRα binds to the AOx-PPRE in a sequence-specific manner. *In vitro* synthesized TRα, RXRα, and PPARα (1 μL each) were coincubated with radiolabeled AOx-PPRE alone (lanes d and h) and in the presence of 10-, 50-, and 100-molar excess of unlabeled, double-stranded nonspecific competitor DNA (5'-GATCCTACCCATACGAC-GTCCCAGACTACGCTTGAGCT and its complement; lanes e-g, respectively) or 10-, 50-, and 100-molar excess of unlabeled AOx-PPRE (lanes i-k, respectively) and analyzed by gel retardation. Lanes a and b are incubations carried out with combinations of *in vitro* translated PPARα and RXRα and of TRα and RXRα (1 μL of each), respectively, to indicate the positions of the heterodimeric complexes. Lane c is a control in which the AOx-PPRE probe was incubated with unprogrammed lysate.

3.2.2 TRα Competes with PPARα for Binding to the AOx-PPRE

When constant amounts of PPARα and RXRα were coincubated with increasing amounts of TRα, the PPARα/RXRα complex gradually disappeared, concomitant with an increase in the intensity of the TRα/RXRα complex and the TRα monomeric complex (Fig. 3-5). This result could be due to a competition of TRα for limiting amounts of RXRα, a competition of TRα/RXRα heterodimers for the AOx-PPRE binding site, and/or the formation of nonbinding TRα/PPARα heterodimers. To address these possibilities, gel electromobility shift analysis was conducted in a reciprocal manner in which constant amounts of TRα were coincubated with increasing amounts of *in vitro* translated RXRα, PPARα, or both receptors (Fig. 3-6). Increasing amounts of RXRα led to an increase in the amount of TRα/RXRα complex, concomitant with a decrease in the amount of TRα monomeric complex (lanes b and c). This result would suggest that RXRα was limiting for complex formation under these conditions. Incubation of TRα with increasing amounts of PPARα led to a decrease in the formation of the TRα monomeric complex, suggesting that PPARα can sequester TRα (compare lane a to lanes d and e). Coincubation of TRα and PPARα with increasing amounts of RXRα led to a reduction in the amount of TRα monomeric complex, concomitant with increased amounts of PPARα/RXRα and TRα/RXRα complexes (lanes f and g). These results suggest that TRα can inhibit binding of PPARα to the AOx-PPRE by competing for the common heterodimerization partner RXRα and by binding to the AOx-PPRE on its own or cooperatively with RXRα. Moreover, the availability of PPARα/RXRα heterodimers capable of binding to the AOx-

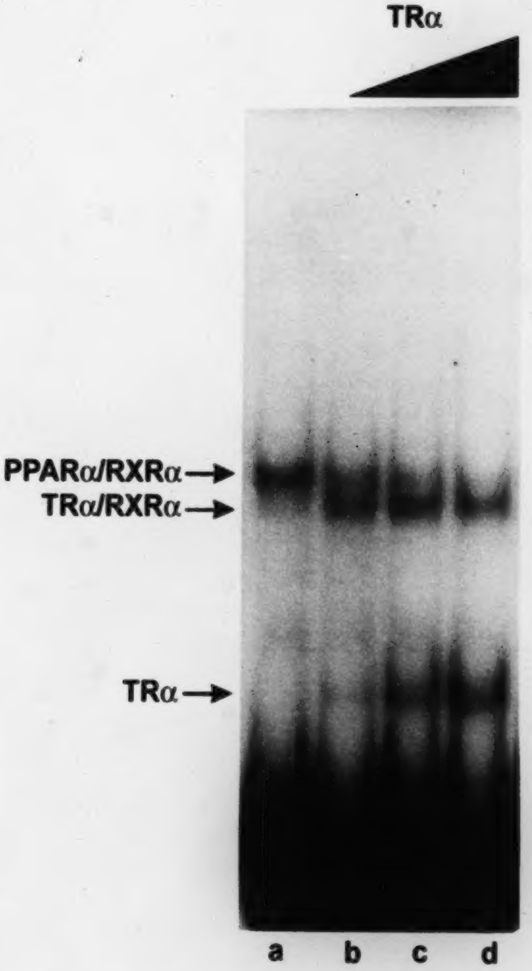


Figure 3-5. TRα competes with PPARα/RXRα for binding to the AOx-PPRE. *In vitro* translated PPARα and RXRα (2 μL of each) were incubated in the absence (lane a) or presence of 2, 4 and 6 μL of *in vitro* translated TRα (lanes b, c and d, respectively) and analyzed by gel retardation. Volumes of lysate were kept constant by the addition of unprogrammed lysate as appropriate. TRα inhibits the formation of the PPARα/RXRα complex concomitant with the formation of the TRα/RXRα complex and the TRα complex.

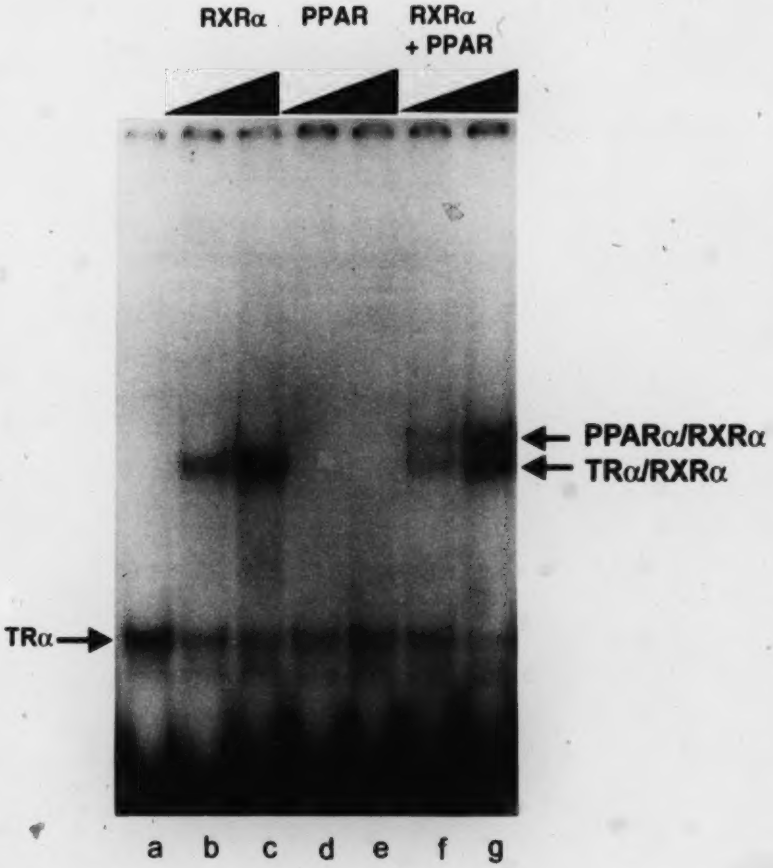

RXRα
PPAR
RXRα
+ PPAR
PPARα/RXRα
TRα/RXRα
TRα
a
b
c
d
e
f
g

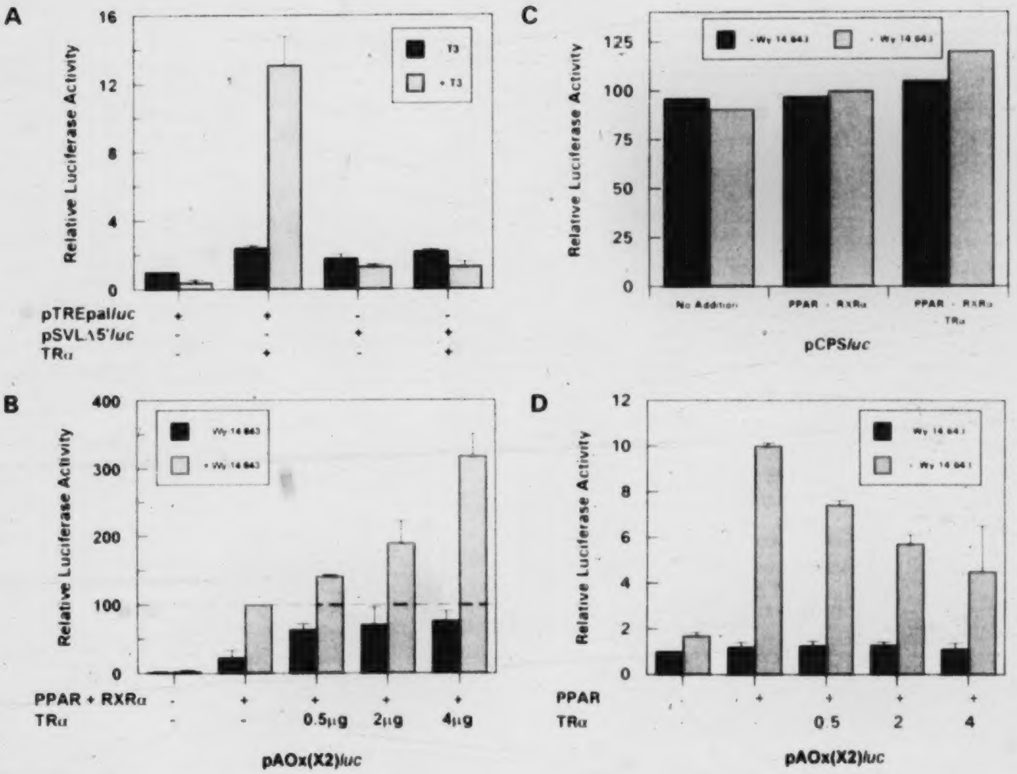
Figure 3-6. Effects of increasing amounts of RXRα and PPARα on the formation of TRα-dependent protein/DNA complexes. *In vitro* translated TRα (2 μL) was mixed with RXRα (2 μL, lane b; 5 μL, lane c), PPARα (2 μL, lane d; 5 μL, lane e), PPARα (1 μL) + RXRα (1 μL) (lane f) or PPARα (1 μL) + RXRα (4 μL) (lane g) in the presence of radiolabeled AOx-PPRE and analyzed by gel retardation. Lysate volumes were kept constant by the addition of unprogrammed lysate as appropriate.

PPRE may also be restricted through the formation of non-PPRE-binding TRα/PPARα complexes.

3.2.3 TRα Stimulates Transactivation by PPARα/RXRα Heterodimers

Transient transfection assays were carried out in BSC40 cells to examine the effects of expression of TRα on PPARα/RXRα-dependent transactivation of a reporter gene plasmid containing two copies of the AOx-PPRE (pAOx(X2)*luc*). Control transfections showed T3-dependent activation of a luciferase reporter gene plasmid containing a palindromic TRE (pTREpal*luc*) following cotransfection with the TRα expression vector (Fig. 3-7, A). Expression from the parental reporter gene plasmid lacking the TRE (pSVΔ5'*luc*) was unaffected by expression of TRα or by the presence of T3, as reported previously (Glass *et al.*, 1988). Figure 3-7, B shows the effects of transfecting increasing amounts of the TRα expression plasmid on transactivation from the AOx-PPRE reporter gene plasmid by PPARα and RXRα . Cotransfection of PPARα and RXRα expression plasmids resulted in an approximately 10-fold activation of expression of the AOx-PPRE reporter gene plasmid over basal levels in the absence of exogenously added PPARα activators, as has been previously demonstrated (Marcus *et al.*, 1993). This activator-independent induction is presumably due to the presence of endogenous PPARα activators. Addition of the potent peroxisome proliferator Wy-14,643 to the transfections resulted in a 50- to 100-fold stimulation of activity from the AOx-PPRE reporter gene plasmid over basal levels. Interestingly, cotransfection with increasing amounts of TRα expression plasmid resulted in progressively enhanced stimulation by PPARα/RXRα

Figure 3-7. TRα enhances transactivation by PPARα/RXRα *in vivo*. (A) BSC40 cells were cotransfected with the reporter plasmids pTREpal*luc* or pSVLΔ5'*luc* and the TRα expression plasmid pRSV-TRα in the presence or absence of T3, as indicated. Luciferase assays were performed as indicated in "Materials and Methods". The values reported are the averages (±SEM) of three independent transfections carried out in duplicate and normalized to the value obtained with pTREpal*luc* alone (taken as 1). (B) BSC40 cells were transfected with pAOx(X2)*luc* in the presence or absence of the peroxisome proliferator Wy-14,643, along with a constant amount of PPARα and RXRα expression plasmids (2 μg each) and increasing amounts of the TRα expression plasmid (in μg), as indicated. Plasmid dosage was normalized in each case with the appropriate amounts of the corresponding empty expression vectors. The values reported are the averages (±SEM) of a minimum of three independent transfections carried out in duplicate and normalized to the value obtained from Wy-14,643-treated cells cotransfected with PPARα and RXRα expression plasmids (taken as 100%). The results show that TRα potentiates Wy-14,643-mediated transactivation by PPARα/RXRα. (C) BSC40 cells were cotransfected with the reporter gene pCPS*luc* and plasmids (2 μg each) expressing the indicated receptors in the presence or absence of Wy-14,643 and T3, as indicated. The values reported are the averages of duplicate transfections and are normalized to the value obtained with pCPS*luc*-cotransfected with PPARα and RXRα expression plasmids in the presence of Wy-14,643 (taken as 100%). Readings of the duplicate samples did not vary by more than 15%. (D) The TRα expression plasmid pRSV-TRα (in μg) was cotransfected with pAOx(X2)*luc* alone or in combination with the PPARα-expression plasmid (2 μg) in the presence of Wy-14,643, as indicated. The values reported are the averages (±SEM) or three independent transfections carried out in duplicate and normalized to the value obtained for cells transfected with the pAOx(X2)*luc* reporter plasmid alone (taken as 1).


A
Relative Luciferase Activity
16
12
8
4
0
- T3
+ T3
pTREpal*luc* + + - -
pSVLΔ5'*luc* - - + +
TRα - + - +
C
Relative Luciferase Activity
125
100
75
50
25
0
- Wy 14 643
+ Wy 14 643
No Addition
PPAR + RXRα
PPAR + RXRα + TRα
pCPS*luc*
B
Relative Luciferase Activity
400
300
200
100
0
- Wy 14 643
+ Wy 14 643
PPAR + RXRα - + + + +
TRα - - 0.5μg 2μg 4μg
pAOx(X2)*luc*
D
Relative Luciferase Activity
12
10
8
6
4
2
0
- Wy 14 643
+ Wy 14 643
PPAR - + + + +
TRα 0.5 2 4
pAOx(X2)*luc*

heterodimers. Cotransfection of up to 4 µg of TRα expression plasmid (representing a 2-fold molar excess over PPARα and RXRα expression plasmids) resulted in a 200- to 300-fold stimulation of Wy-14,643-dependent induction by PPARα/RXRα, and to a lesser extent (40- to 50-fold stimulation over basal) in the absence Wy-14,643. To examine the requirements of a PPRE for TRα-dependent stimulation, cotransfection experiments similar to those reported above were carried out with pCPS*luc*, the parental vector used to construct pAOx(X2)*luc*. Transfection of PPARα/RXRα alone, or in combination with TRα, had little effect on the expression of this reporter gene construct, either in the absence or presence of Wy-14,643 (Fig. 3-7, C). Transfection of the PPARα expression vector alone resulted in a modest (10-fold) stimulation of the AOx-PPRE reporter gene activity in the presence of Wy-14,643, likely because of interaction between PPARα and endogenous RXRα and/or other cofactors (Fig. 3-7, D). However, TRα did not further stimulate PPAR-mediated activation under these conditions. Indeed, activation was reduced by approximately 50% when a 2-fold excess of TRα expression plasmid was transfected. Therefore, TRα-mediated stimulation of Wy-14,643-dependent transcriptional induction also requires the exogenous addition of both PPARα and RXRα.

3.3 Discussion

Several groups have indicated that PPARα can differentially influence TRα-mediated activation of thyroid-hormone responsive genes (Bogazzi *et al.*, 1994; Jow and Mukherjee, 1995; Meier-Heusler *et al.*, 1995; Juge-Aubry *et al.*, 1995). The findings presented in this chapter show that TRα modulates PPARα-mediated transactivation of peroxisome proliferator responsive genes *in vivo* and can disrupt the binding of PPARα/RXRα heterodimers to PPREs *in vitro*. Thus, crosstalk and coupling between the thyroid hormone and peroxisome proliferator signaling pathways are important to the reciprocal regulation of both thyroid hormone- and peroxisome proliferator-responsive genes.

The data presented in this chapter show that TRα is capable of binding to the AOx-PPRE in a sequence specific manner on its own as well as a heterodimer with RXRα. This is not necessarily unexpected, since, in addition to response elements containing half sites that are configured in a DR4 arrangement, thyroid hormone receptors bind promiscuously as monomers or as homo- and heterodimers to a wide variety of structurally diverse response elements configured in different manners (Desvergne, 1994). Therefore, the AOx-PPRE defines a novel target for TRα. This finding, coupled with previous observations that COUP-TFI (Miyata *et al.*, 1993) and HNF-4 (Winrow *et al.*, 1994) also bind to the AOx-PPRE, provides further evidence that the AOx-PPRE is a composite response element that is a target for multiple members of the nuclear receptor superfamily.

The binding of TRα and of TRα/RXRα to the AOx-PPRE led to a decrease in the binding of PPARα/RXRα to this element. The findings presented suggest that this decrease was the net result of TRα competing for the common heterodimerization partner RXRα and for binding to the AOx-PPRE. Moreover, as shown by others, PPARα and TRβ can heterodimerize in solution, implying that TRα, likely sequesters PPARα as well as RXRα (Bogazzi *et al.*, 1994). However, TRα/PPARα was unable to bind to the AOx-PPRE, and a natural DNA-binding site for the TRα/PPARα heterodimer pair, if it exists, remains to be identified. Interestingly, the TRβ/PPARα heterodimer has been shown to be capable of binding to a DR2 element present in the promoter region of the gene encoding myelin proteolipid protein (Bogazzi *et al.*, 1994).

The results showing that TRα augments PPARα/RXRα-mediated transactivation from the AOx-PPRE *in vivo* contrasts with the *in vitro* results showing that TRα inhibits the binding of PPARα/RXRα to the AOx-PPRE. It is possible that TRα may not bind to the AOx-PPRE *in vivo*, perhaps because there exist additional cellular factors that stabilize PPARα/RXRα interactions on the AOx-PPRE. TRα-dependant stimulation of PPARα/RXRα activity *in vivo* may thus be manifested through mechanisms that do not involve the binding of TRα to the AOx-PPRE. For instance, it may be possible that TRα titrates, and therefore counteracts, the effects of a repressor that attenuates transcriptional activation by PPARα/RXRα heterodimers, thereby relieving repression. Consistent with this possibility, TRα has been shown to bind to an inhibitory factor that also interacts with the retinoic acid receptor alpha, RARα (Casanova *et al.*, 1994). Therefore, overexpression of TRα may lead to an alteration in the repertoire of RXRα available for interaction with PPARα.

The fact that the AOx-PPRE reporter gene construct contains a tandem repeat of the PPRE offers an alternative explanation for our finding that TRα augments PPARα/RXRα-mediated transactivation from the AOx-PPRE *in vivo*. It may be possible that TRα homodimers or TRα/RXRα heterodimers simultaneously occupy the duplicated element along with bound PPARα/RXRα, thereby acting synergistically to enhance transcriptional activation.

3.4 Summary

The results of the data presented suggest that there is signaling crosstalk between PPARα and TRα that may be physiologically important in the regulation of lipid homeostasis and in mediating the thyromimetic effects of peroxisome proliferators. The mechanism by which TRα and PPARα differentially modulate transactivation by the other is complex and appears to be dependent upon a dynamic balance amongst RXRα, PPARα, TRα, and possibly other cellular cofactors, including coactivators, corepressors, and nuclear hormone receptors.

CHAPTER 4

The Orphan Nuclear Hormone Receptor RevErbα Modulates Expression from the Promoter of the Hydratase-Dehydrogenase Gene by Inhibiting Peroxisome Proliferator Activated Receptor α-Dependent Transactivation[§]

[§] A version of this chapter has been published. Kassam, A., Capone, J.P., and Rachubinski, R. A. *J. Biol. Chem.* 274:22895-22990 (1999). Used with permission from The American Society for Biochemistry and Molecular Biology.

4.1 Abstract

Considering the similarities between RevErbα and PPARα with respect to their consensus half-sites, tissue distribution, response to fibrates, and putative biological functions, we investigated a possible role for RevErbα in lipid metabolism as an upstream regulatory factor for the peroxisomal β-oxidation pathway. Here we demonstrate that the orphan nuclear hormone receptor, RevErbα, modulates PPARα/RXRα-dependent transactivation in a response element-specific manner. *In vitro* binding analysis showed that RevErbα bound the HD-PPRE but not the AOx-PPRE. Determinants within the HD-PPRE required for RevErbα binding were distinct from those required for PPARα/RXRα binding. In transient transfections, RevErbα antagonized transactivation by PPARα/RXRα from an HD-PPRE luciferase reporter construct, whereas no effects were observed with an AOx-PPRE reporter construct. These data identify the HD gene as a target for RevErbα and illustrate crosstalk between the RevErbα and PPARα signaling pathways on the HD-PPRE. Our results suggest a novel role for RevErbα in regulating peroxisomal β-oxidation.

4.2 Results

4.2.1 RevErbα Binds to the HD-PPRE

Both the HD- and AOx-PPREs contain potential binding sites for RevErbα (Fig.4-1, A). We therefore examined whether RevErbα is capable of binding to the HD- and AOx-PPREs by performing electromobility shift analysis (EMSA) with radiolabeled PPRE probes and *in vitro* translated receptors. As expected, PPARα and RXRα bound as a

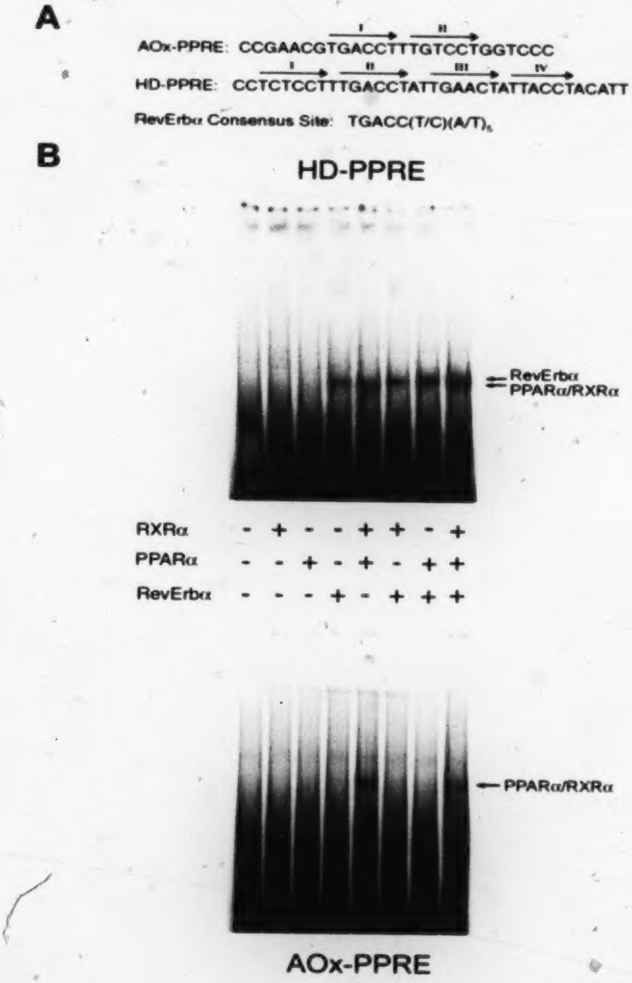

A
I
II
AOx-PPRE: CCGAACGTGACCTTTGTCCTGGTCCC
I
II
III
IV
HD-PPRE: CCTCTCCTTTGACCTATTGAACTATTACCTACATT
RevErbα Consensus Site: TGACC(T/C)(A/T)6
B
HD-PPRE
RevErbα
PPARα/RXRα
RXRα - + - - + + - +
PPARα - - + - + - + +
RevErbα - - - + - + + +
PPARα/RXRα
AOx-PPRE

Figure 4-1. RevErbα binds to the HD-PPRE but not the AOx-PPRE. (A), comparison of the sequences of the AOx- and HD-PPREs to the consensus RevErbα binding site. *Arrows* and *Roman numerals* indicate the locations and directions of the TGACCT-like motifs. (B) *In vitro* synthesized PPARα (1 μL), RXRα (1 μL), and RevErbα (4 μL) were incubated alone or in combination with radiolabeled HD-PPRE (*upper panel*) or AOx-PPRE (*lower panel*) probe, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed on 3.5% polyacrylamide gels. The positions of the heterodimeric PPARα/RXRα and monomeric RevErbα complexes are indicated by *arrows*.

heterodimer on the HD-PPRE (Fig. 4-1, B). RevErbα also bound the HD-PPRE, forming a complex with a mobility slightly less than that formed by the PPARα/RXRα heterodimer. Inclusion of RXRα or PPARα with RevErbα in the binding reactions had no effect on the formation of the RevErbα/HD-PPRE complex. Moreover, coincubation of all three receptors with the HD-PPRE produced only two distinct complexes corresponding to PPARα/RXRα heterodimers and RevErbα monomers (Fig. 4-1, B, *far right lane*). Therefore, the three receptors do not co-occupy the HD-PPRE in some higher order complex, and RevErbα does not form complexes with PPARα or RXRα on this element *in vitro* under the EMSA conditions used. In contrast to the results obtained with the HD-PPRE, RevErbα was unable to bind to the AOx-PPRE. Furthermore, only the characteristic PPARα/RXRα heterodimer was generated on the AOx-PPRE when RevErbα was coincubated with PPARα and RXRα (Fig. 4-1, B). Binding of RevErbα to the HD-PPRE was specific, since the radiolabeled complex was refractory to competition by nonspecific unlabeled oligonucleotide but was eliminated by addition of unlabeled HD-PPRE oligonucleotide (Fig. 4-2).

We next investigated whether binding by PPARα/RXRα to the HD-PPRE could be influenced by RevErbα and *vice versa*. EMSA was first performed using radiolabeled HD-PPRE, constant amounts of PPARα/RXRα, and increasing amounts of RevErbα (Fig. 4-3, A). Increasing the amount of RevErbα did not affect binding of PPARα/RXRα to the HD-PPRE, but did result in greater amounts of RevErbα monomeric complex forming on the HD-PPRE. Conversely, when EMSA was performed with a constant amount of RevErbα, increasing the amounts of PPARα and RXRα led to increased formation of

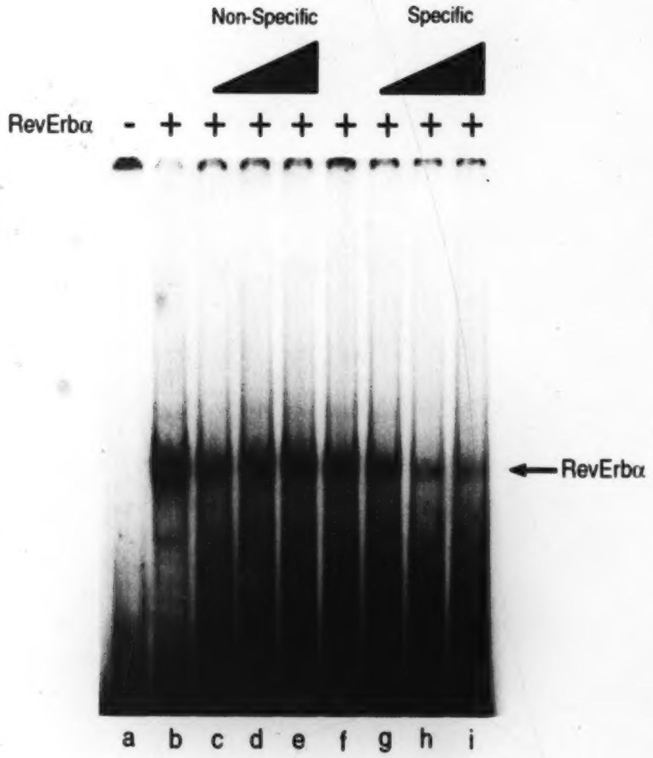


Figure 4-2. Binding of RevErbα is sequence-specific. EMSA was performed with *in vitro* synthesized RevErbα and radiolabeled HD-PPRE probe. Volumes were kept constant by addition of unprogrammed lysate. Competition experiments were performed by addition of 10-, 50- or 100-fold molar excess of unlabeled competitor DNA (5'-GATCCCGTGCATGCTAATGATATTCT and its complement) (*Nonspecific*) or unlabeled HD-PPRE (*Specific*). The position of the RevErbα complex is indicated.

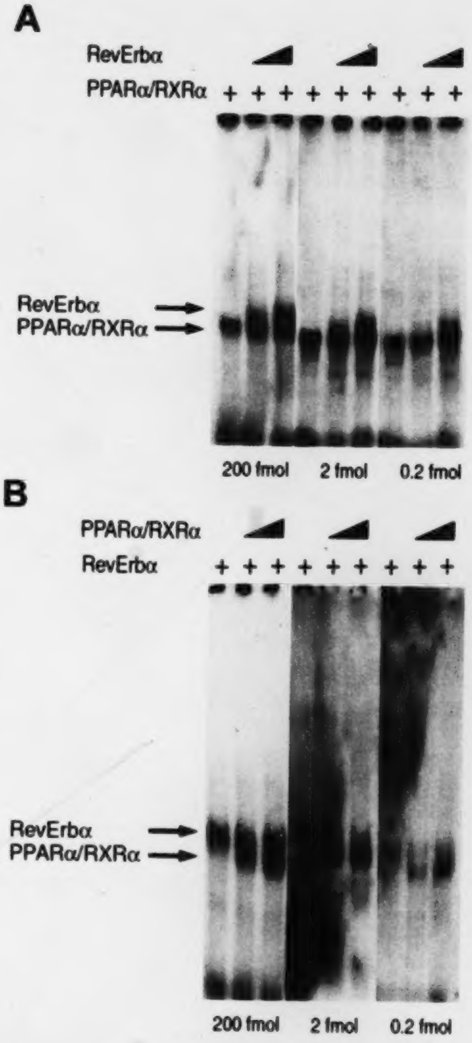

A
RevErbα
PPARα/RXRα + + + + + + + + +
RevErbα
PPARα/RXRα
200 fmol
2 fmol
0.2 fmol
B
PPARα/RXRα
RevErbα + + + + + + + + +
RevErbα
PPARα/RXRα
200 fmol
2 fmol
0.2 fmol

Figure 4-3. RevErbα and PPARα/RXRα bind independently to the HD-PPRE. Binding reactions contained *in vitro* synthesized PPARα/RXRα (0.5 μL of each) and RevErbα (5 or 10 μL) (A), or RevErbα (10 μL) and PPARα/RXRα (1 or 2 μL of each) (B). Radiolabeled HD-PPRE probe was added at 200, 2, or 0.2 fmol, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed on 2.5% polyacrylamide gels. Autoradiography was for 16 h (200 fmol, A and B), 6 d (2 fmol, A), 3 d (2 fmol, B), 14 d (0.2 fmol, A), or 7 d (0.2 fmol, B). *Arrows* indicate the positions of the PPARα/RXRα heterodimer and the RevErbα monomer.

PPARα/RXRα complexes on the HD-PPRE, while the binding of RevErbα monomers was unaffected (Fig. 4-3, B). Similar findings were observed over a wide range of HD-PPRE probe concentration (Fig. 4-3, A and B). These results suggest that RevErbα and PPARα/RXRα bind independently to the HD-PPRE, and that RevErbα monomers and PPARα/RXRα heterodimers do not simultaneously occupy the HD-PPRE.

4.2.2 RevErbα and PPARα/RXRα Bind to Distinct Sites on the HD-PPRE

The HD-PPRE consists of four half-sites (sites I-IV) related to the consensus TGACCT/C hexameric half-site. Each half-site of the HD-PPRE could potentially serve as a binding site for RevErbα. In order to determine the sequence requirements for RevErbα interaction, oligonucleotide probes harboring mutations in each of the four hexameric half-sites of the HD-PPRE were used in binding studies (Fig. 4-4). EMSA showed that mutations in sites I, III, and IV did not affect binding of RevErbα, either in the absence (Fig. 4-5) or presence of PPARα/RXRα (Fig. 4-4). However, disruption of site II effectively abrogated RevErbα binding. This observation is consistent with the fact that site II most closely resembles the consensus sequence for RevErbα binding (Fig. 4-1, A). Binding of PPARα/RXRα was found to require the integrity of sites III and IV but not of sites I and II (Fig. 4-4), as previously demonstrated (Winrow *et al.*, 1998; Zhang *et al.*, 1993). These results show that RevErbα and PPARα/RXRα target distinct half-sites on the HD-PPRE.

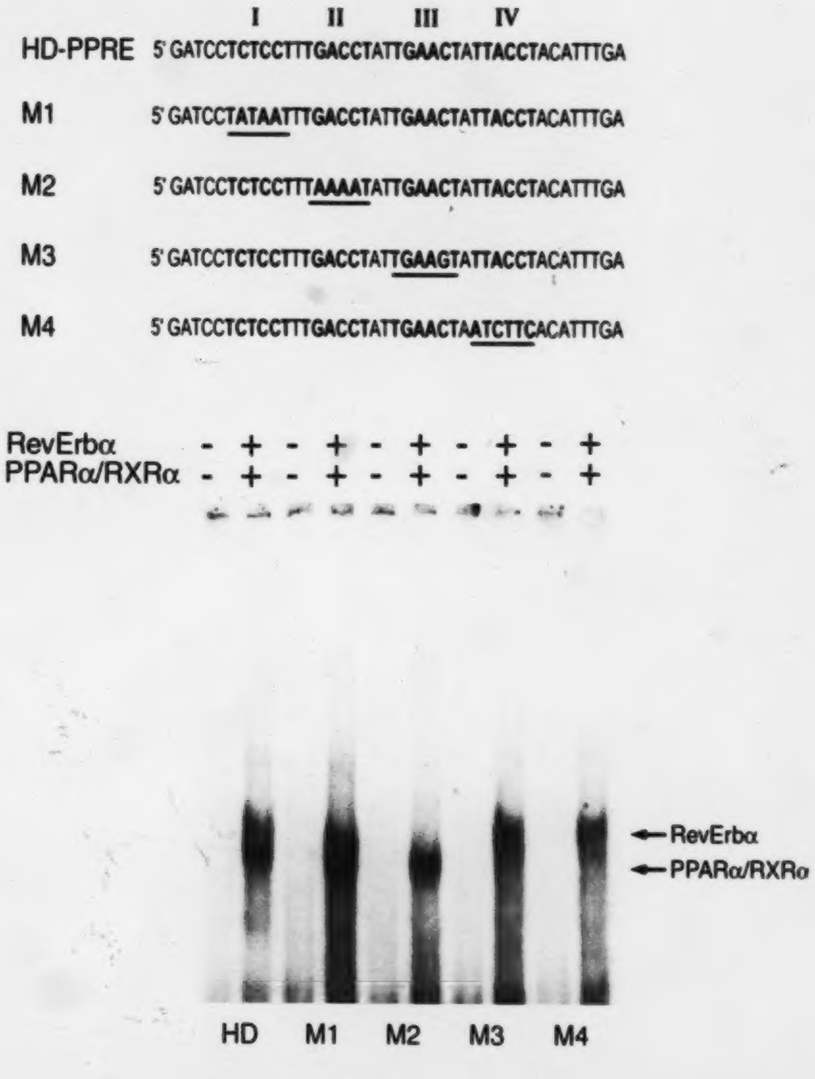

I
II
III
IV
HD-PPRE 5' GATCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA
M1 5' GATCCTATAATTTGACCTATTGAACTATTACCTACATTTGA
M2 5' GATCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA
M3 5' GATCCTCTCCTTTGACCTATTGAAGTATTACCTACATTTGA
M4 5' GATCCTCTCCTTTGACCTATTGAACTAATCTTCACATTTGA
RevErbα - + - + - + - + - +
PPARα/RXRα - + - + - + - + - +
RevErbα
PPARα/RXRα
HD
M1
M2
M3
M4

Figure 4-4. RevErbα and PPARα/RXRα bind to distinct sites within the HD-PPRE. Sequences of the wild-type HD-PPRE and of the oligonucleotides M1, M2, M3, and M4 containing mutations (*underlined*) in the consensus hexameric half-sites I, II, III, and IV (*bold*), respectively. EMSA was performed on a 2.5% polyacrylamide gel with *in vitro* synthesized PPARα/RXRα (1 μL of each) and RevErbα (10 μL) and wild-type or mutant HD-PPRE probes, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. *Arrows* show positions of bound complexes.

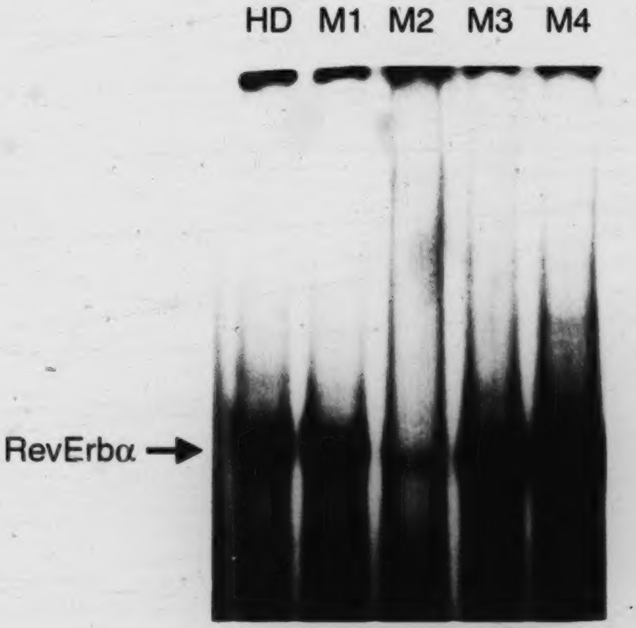


Figure 4-5. Mutation of site II of the HD-PPRE abolishes RevErbα binding. EMSA was performed as in Figure 4-4. Binding reactions contained 10 µL of *in vitro* synthesized RevErbα and wild-type or mutant HD-PPRE probes, as indicated. The position of the RevErbα complex is indicated.

4.2.3 RevErbα Antagonizes Transactivation by PPARα/RXRα from the HD-PPRE but Not the AOx-PPRE

To investigate the *in vivo* properties of RevErbα on transcriptional regulation, we carried out transient transfection assays using luciferase reporter plasmids containing either the HD-PPRE (pHD(X3)*luc*) or the AOx-PPRE (pAOx(X2)*luc*), along with expression plasmids for RevErbα, PPARα, and/or RXRα in BSC40 African monkey kidney cells. Cotransfection of PPARα and RXRα with the HD-PPRE reporter plasmid led to a 2-fold induction of transcription over basal levels in the absence of the peroxisome proliferator, Wy-14,643 (Fig. 4-6, A). Addition of proliferator led to a potent induction of transcription (10- to 15-fold) over basal levels. Increasing amounts of RevErbα expression plasmid inhibited transactivation from the HD-PPRE by PPARα/RXRα both in the presence and absence of peroxisome proliferator. Transactivation by PPARα/RXRα was reduced by 80% at the highest amount of RevErbα expression plasmid used (2 μg). PPARα/RXRα also activated transcription of a reporter gene that contained the HD-PPRE harboring a disruption in site II; however, in this case, RevErbα-dependent inhibition was not observed (Fig. 4-6, B). This finding is in agreement with *in vitro* binding data for RevErbα (see Fig. 4-4) and indicates that inhibition of PPARα/RXRα-mediated activation is dependent upon RevErbα binding to the HD-PPRE.

RevErbα did not significantly affect transcriptional activation by PPARα/RXRα on the AOx-PPRE, in either the absence or presence of Wy-14,643 (Fig. 4-6, C). These data are in keeping with *in vitro* binding data showing that RevErbα failed to bind the AOx-PPRE (Fig. 4-1, B). Control transfections with the parental reporter construct

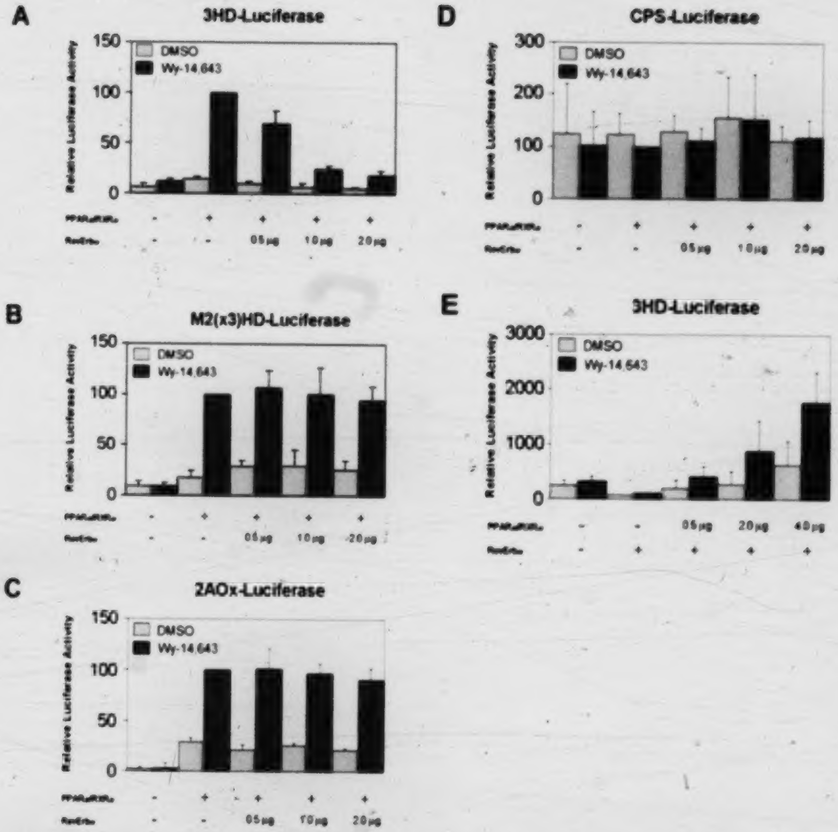


Figure 4-6. RevErbα antagonizes transactivation from the HD-PPRE by exogenous PPARα/RXRα, whereas exogenous expression of PPARα/RXRα can overcome transcriptional repression by RevErbα on the HD-PPRE. BSC40 monolayer cells were transfected with 5 μg of the luciferase reporter pHD(X3)*luc* (A), pM2(X3)*luc* (B), pAOx(X2)*luc* (C), or pCPS*luc* (D), plasmids for PPARα (2 μg), RXRα (2 μg), and RevErbα (0.5 - 2 μg), in the absence or presence of 0.1 mM Wy-14,643. Plasmid dosage was normalized by addition of empty expression vector. Cells were harvested 48 h post-transfection, and luciferase activity quantitated. Transfections were carried out in duplicate and represent the average (± SEM) of three independent experiments. Values presented are relative to the value obtained for cells transfected with PPARα and RXRα in the presence of Wy-14,643 (taken as 100%). (E) BSC40 cells were transfected with 5 μg of the luciferase reporter pHD(X3)*luc* and expression plasmids for RevErbα (2 μg) and PPARα/RXRα (0.5 - 4 μg) in the absence or presence of 0.1 mM Wy-14,643. Plasmid dosage was normalized by the addition of empty expression vector. Transfections were carried out in duplicate and represent the average (± SEM) of three independent experiments. Values presented are relative to the value obtained for cells transfected with RevErbα (2 μg) alone in the presence of Wy-14,643 (taken as 100%).

pCPS*luc*, which lacks a PPRE, showed that the presence of RevErbα, PPARα, and RXRα did not influence basal levels of luciferase activity (Fig. 4-6, D), demonstrating the need for a functional PPRE for receptor activity. Together, these data show that RevErbα antagonizes transactivation by PPARα/RXRα specifically from the HD-PPRE.

4.2.4 Increased Amounts of PPARα and RXRα Can Overcome Repression by RevErbα

We were interested in determining whether increased amounts of PPARα and RXRα could modulate the repression exerted by RevErbα on transcription from the HD-PPRE. Transient transfections with a constant amount of RevErbα expression plasmid and increasing amounts of PPARα and RXRα expression plasmids demonstrated that the repressive effects of RevErbα could be alleviated in a dose-dependent manner by increasing amounts of PPARα and RXRα, either in the presence or absence of Wy-14,643 (Fig. 4-6, E). These results suggest that the net transcriptional response from the HD-PPRE is influenced by the relative levels of PPARα, RXRα and RevErbα *in vivo*.

4.3 Discussion

A number of recent observations have pointed to possible interplay between the RevErbα and PPARα signaling pathways. First, there is strong sequence similarity between the RevErbα consensus binding site and the PPREs of the AOx and HD genes. Second, a role for RevErbα in PPAR-mediated signaling has been suggested by studies showing that the PPRE of the CYP4A6 gene binds both RevErbα and PPARα (Hsu *et al.*, 1998) and that the PPARγ isoform, a key regulator of adipogenesis, may in turn be regulated by RevErbα, whose mRNA levels are dramatically increased during differentiation of preadipocytes to adipocytes (Chawla and Lazar, 1993). Third, RevErbα has been shown to be encoded on the opposite strand of the gene encoding thyroid hormone receptor α and to be able to bind the thyroid hormone, triiodothyronine (Miyajima *et al.*, 1989), and we have previously demonstrated crosstalk between thyroid hormone receptor α and PPARα in regulating transcription from the AOx-PPRE (Chapter 3). As a result of these observations, we considered the possibility of a role for RevErbα in regulating transcription from the AOx- and HD-PPREs.

We have demonstrated here that the RevErbα and PPARα signaling pathways converge and that RevErbα serves to repress transcriptional activation specifically from the HD-PPRE. Interestingly, RevErbα had no effect on PPARα/RXRα-mediated activation via the AOx-PPRE. Consistent with this observation, RevErbα was shown to bind specifically to the HD-PPRE but not the AOx-PPRE. The HD-PPRE is comprised of four hexameric direct repeats arranged as two tandem DR1 arrays separated by two nucleotides (a DR2). This complex arrangement is thought to permit the interaction of a diverse array of nuclear hormone receptors with the HD-PPRE, thereby increasing the complexity of transcriptional regulation from this PPRE. RevErbα and PPARα/RXRα target distinct half sites on the HD-PPRE. The integrity of site II is required for RevErbα binding, while sites III/IV serve to bind RXRα/PPARα. Site II was also required for the RevErbα-dependent repressive effects on transcriptional activation by PPARα/RXRα, indicating that inhibition requires binding of RevErbα to the HD-PPRE. Although sites III/IV have been shown to be essential and sufficient for PPARα/RXRα binding and activity (Winrow *et al.*, 1998; Zhang *et al.*, 1993), an arrangement in which PPARα/RXRα heterodimers are bound to both DR1 sites has been suggested to yield the highest level of transactivation (Chu *et al.*, 1995). Since RevErbα occupies site II within the HD-PPRE, this may preclude binding of PPARα/RXRα to the upstream DR1 element, resulting in reduced levels of transactivation from the HD-PPRE by PPARα/RXRα.

Although RevErbα and PPARα/RXRα use distinct determinants on the HD-PPRE, we did not observe a higher order complex containing all three receptors *in vitro*. However, the limitation of our *in vitro* binding analysis does not preclude the possibility of a higher order complex forming among RevErbα, PPARα and RXRα *in vivo*, perhaps through the cooperativity or association of regulatory cofactors such as SRC-1, p300, N–CoR and SMRT-1 (Chen and Evans, 1995; Hörlein *et al.*, 1995; DiRenzo *et al.*, 1997; Dowell *et al.*, 1997). The involvement of such cofactors in transcriptional regulation by nuclear hormone receptors is well established. Indeed, NCoR, SMRT and SUNCoR have been shown to interact with RevErbα in mammalian cells causing transrepression (Downes *et al.*, 1996; Zamir *et al.*, 1996, 1997; Burke *et al.*, 1998). A model can be proposed in which repression of transactivation by RevErbα is the result of a shift from active to repressive states of the receptor through its association with corepressors and dissociation from coactivators, respectively. RevErbα can also be envisioned to be subjected to post-translational modifications *in vivo*, such as phosphorylation that could initiate its repressive state. Interestingly, the amino-terminal of RevErbα contains numerous serine and threonine residues that could potentially be phosphorylated (Miyajima *et al.*, 1989).

The ligand for RevErbα remains unknown. It is therefore impossible to ascertain at this time whether an endogenous ligand exists in BSC40 cells that could induce transcriptional repression upon binding RevErbα, as has been demonstrated for androstanol and the mCARβ receptor (Forman *et al.*, 1998). It has been suggested that RevErbα lacks the AF2 transactivation domain that is responsible for ligand binding (Durand *et al.*, 1994), thus precluding the possibility of RevErbα having any capacity for ligand-dependent activation or repression (Zamir *et al.*, 1996). Orphan receptors lacking AF2 domains could instead act as competitors for ligand-inducible receptors (Durand *et al.*, 1994), and such a scenario has been proposed to explain the blocking of RZRα-mediated transactivation by RevErbα (Forman *et al.*, 1994; Retnakaran *et al.*, 1994). Nevertheless, the absence of an AF2 domain does not preclude the possibility that RevErbα contains undetected, and yet undefined, activation domains that could be revealed through interaction with a novel ligand (Harding and Lazar, 1995). RevErbα could also potentially activate transcription by cooperative interaction with a non-AF2-dependent coactivator or, indirectly, by recruiting corepressors away from other nuclear receptors (Zamir *et al.*, 1996). TRβ and PPARα have been reported to form nonbinding heterodimers *in vivo* (Bogazzi *et al.*, 1994), and RevErbα could similarly form inactive, nonbinding complexes (Harding and Lazar, 1995) with PPARα, RXRα or other nuclear receptors, effectively sequestering these receptors and preventing them from forming heterodimers that normally potentiate transcription, leading to an overall repression of transcription. However, we consider the latter scenario unlikely, since inhibition by RevErbα required the integrity of the HD-PPRE and had no effect on PPARα/RXRα-mediated transactivation via the AOx-PPRE.

4.4 Summary

In summary, our results identify the gene encoding enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, the second enzyme of the peroxisomal β-oxidation pathway, as a target for RevErbα and demonstrate that this orphan nuclear hormone receptor serves as a negative modulator of PPARα/RXRα-mediated transactivation from the PPRE of this gene. The repressive effects of RevErbα on the HD-PPRE can be overcome by increasing the concentration of PPARα/RXRα, and together, or results illustrate a convergence of the RevErbα and PPARα signaling pathways in gene regulation. Transcriptional control of peroxisomal β-oxidation involves a complex network of interacting regulatory factors that integrate a diverse array of host signaling pathways to determine the net transcriptional response to a particular environmental or physiological cue.

CHAPTER 5

The Peroxisome Proliferator Response Element of the Gene Encoding the Peroxisomal β-oxidation Enzyme Enoyl-CoA Hydratase/3-Hydroxyacyl-CoA Dehydrogenase Is a Target for Constitutive Androstane Receptor β/9-*cis*-Retinoic Acid Receptor α-Mediated Transactivation[§]

[§] A version of this chapter has been published. Kassam, A., Winrow, C. J., Fernandez-Rachubinski, F., Capone, J. P., and Rachubinski, R. A. *J. Biol. Chem.* 275:4345-4350 (2000). Used with permission from The American Society for Biochemistry and Molecular Biology.

5.1 Abstract

The impetus to investigate the potential for CARβ to regulate expression from the PPREs of genes involved in peroxisomal β-oxidation stems from the similar physiological properties shared between CARβ and PPARα. These include the compositions of their respective response elements, liver expression pattern, and their ability to heterodimerize with RXRα. Here we demonstrate that the HD-PPRE is also a target for the constitutive androstane receptor β (CARβ). *In vitro* binding analysis showed that CARβ bound the HD-PPRE, but not the AOx-PPRE, as a heterodimer with RXRα. Binding of CARβ/RXRα to the HD-PPRE occurred via determinants that overlap partially with those required for PPARα/RXRα binding. *In vivo*, CARβ/RXRα activated transcription from an HD-PPRE luciferase reporter construct. Interestingly, CARβ was shown to also modulate PPARα/RXRα-mediated transactivation in a response element-specific manner. In the presence of the peroxisome proliferator, Wy-14,643, CARβ had no effect on PPARα/RXRα-mediated transactivation from the HD-PPRE, but antagonized transactivation from the AOx-PPRE in both the presence and absence of proliferator. Our results illustrate that transcription of the AOx and HD genes is differentially regulated by CARβ and that the HD gene is a specific target for regulation by CARβ. Overall, this study proposes a novel role for CARβ in the regulation of peroxisomal β-oxidation.

5.2 Results

5.2.1 CARβ Binds to the HD-PPRE as a Heterodimer with RXRα

Both the AOx- and HD-PPREs contain arrays of the consensus hexameric binding motif TGACCT that could potentially serve as binding sites for CARβ. To illustrate the binding capability of CARβ to these response elements, we performed EMSA with radiolabeled PPRE probes and *in vitro* translated receptors. As previously shown (Winrow *et al.*, 1998; Chapters 3 and 4), PPARα and RXRα bound as a heterodimer to the HD-PPRE (Fig. 5-1, A). CARβ did not bind alone to the HD-PPRE but was capable of binding as a heterodimer with RXRα, generating a complex with slightly faster mobility than that of PPARα/RXRα (Fig. 5-1, A). Coincubation of PPARα, RXRα and CARβ with the HD-PPRE produced two distinct complexes corresponding to PPARα/RXRα and CARβ/RXRα heterodimers (Fig. 5-1, A; *far right lane*). No higher order ternary complex was observed, suggesting the lack of cooperativity of all three receptors in binding to the HD-PPRE. In contrast to the results obtained with the HD-PPRE, binding of CARβ as a heterodimer with RXRα on the AOx-PPRE was not observed (Fig. 5-1, B). Binding of CARβ/RXRα to the HD-PPRE was specific, since the radiolabeled complex was refractory to competition by nonspecific unlabeled oligonucleotide but was effectively competed out by the addition of unlabeled HD-PPRE oligonucleotide (Fig. 5-2).

5.2.2 CARβ/RXRα Recognizes the DR2 Element of the HD-PPRE

The HD-PPRE consists of four consensus hexameric TGACCT half-sites (I-IV) in an arrangement of two DR1 elements separated by two base pairs, thereby forming an internal DR2 element (Fig. 5-3). Oligonucleotide probes harboring mutations in each of the four half-sites were used in binding studies to determine which half-sites are responsible for CARβ/RXRα binding. PPARα/RXRα requires the integrity of half-sites

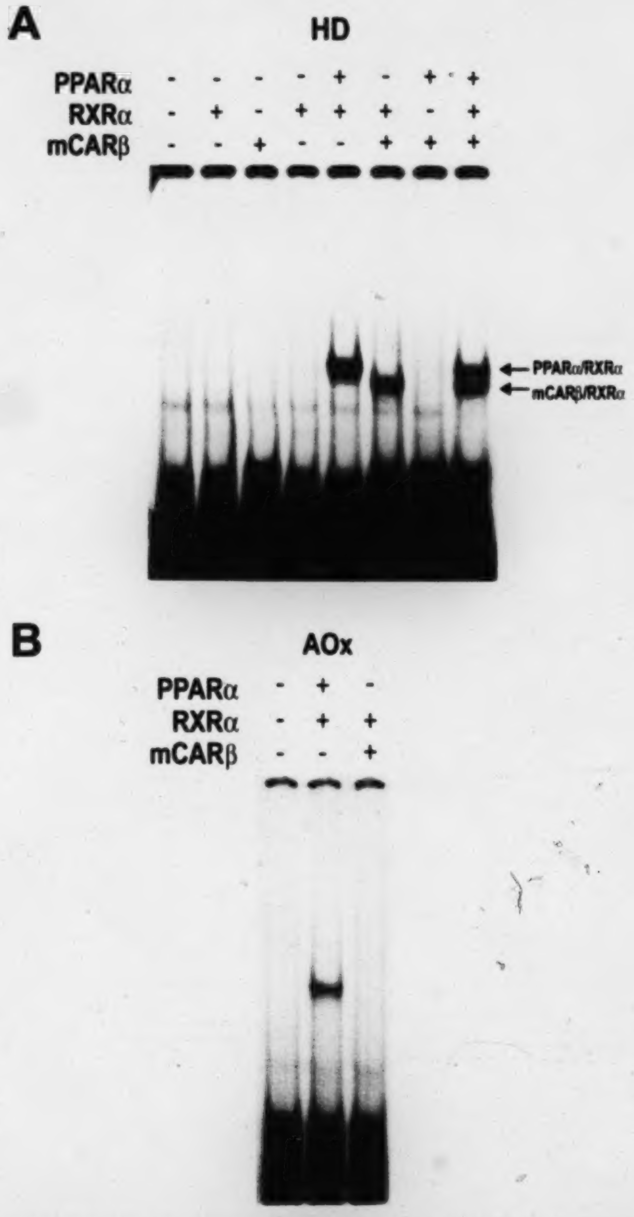

A
HD
PPARα - - - - + - + +
RXRα - + - + + + - +
mCARβ - - + - - + + +
PPARα/RXRα
mCARβ/RXRα
B
AOx
PPARα - + -
RXRα - + +
mCARβ - - +

Figure 5-1. CARβ binds as a heterodimer with RXRα to the HD-PPRE but not the AOx-PPRE. *In vitro* synthesized PPARα, RXRα and CARβ were incubated alone or in combination with radiolabeled HD-PPRE (A) or AOx-PPRE (B). The total amount of lysate in each reaction was kept constant by the addition of unprogrammed lysate. EMSA was performed on 3.5% polyacrylamide gels. The positions of the heterodimeric PPARα/RXRα and CARβ/RXRα complexes are indicated.

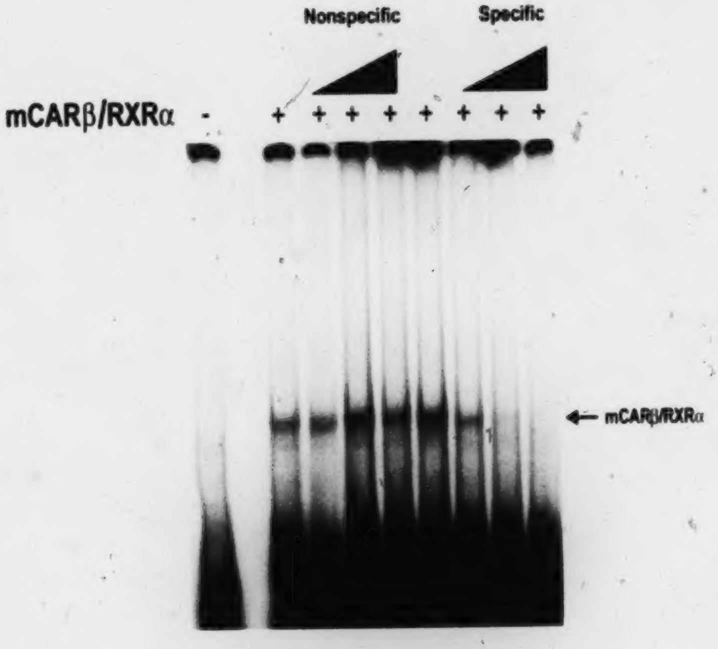


Figure 5-2. Binding of CARβ/RXRα to the HD-PPRE is sequence-specific. EMSA was performed with *in vitro* synthesized CARβ and RXRα and radiolabeled HD-PPRE probe, as indicated. Volumes were kept constant by addition of unprogrammed lysate. Competition experiments were performed by addition of 10-, 50- or 100-fold molar excess of unlabeled competitor DNA (5'-GATCCCGTGCATGCTAATGATATTCT and its complement) (*Nonspecific*) or unlabeled HD-PPRE (*Specific*). The position of the CARβ/RXRα complex is indicated.

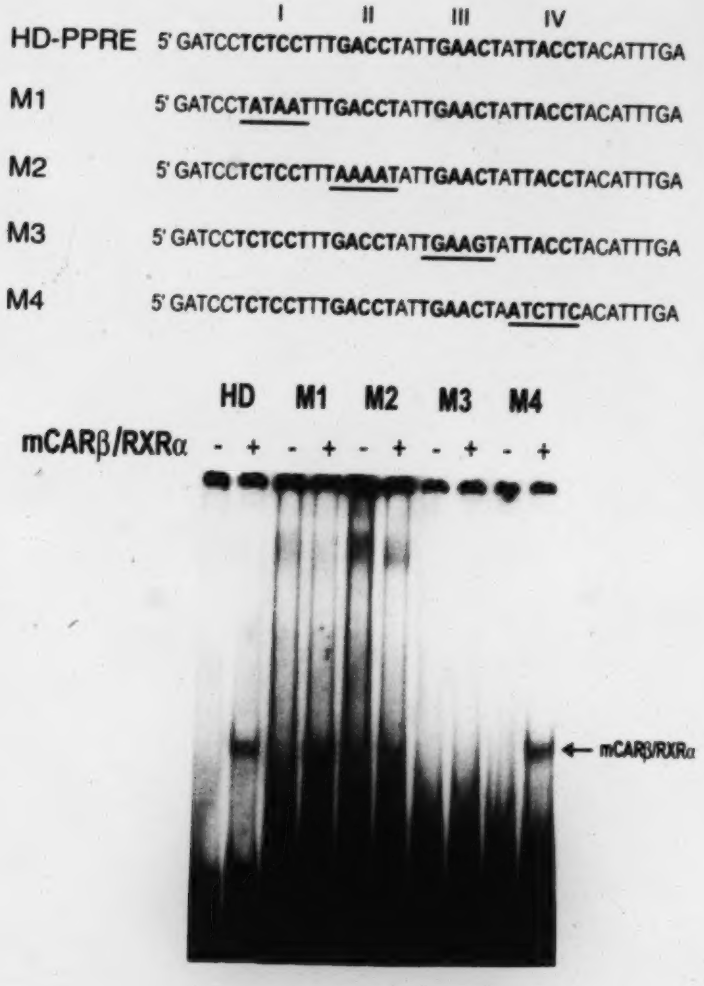

I
II
III
IV
HD-PPRE 5' GATCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA
M1 5' GATCCTATAATTTGACCTATTGAACTATTACCTACATTTGA
M2 5' GATCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA
M3 5' GATCCTCTCCTTTGACCTATTGAAGTATTACCTACATTTGA
M4 5' GATCCTCTCCTTTGACCTATTGAACTAATCTTCACATTTGA
HD
M1
M2
M3
M4
mCARβ/RXRα
- + - + - + - + - +
mCARβ/RXRα

Figure 5-3. CARβ/RXRα recognizes the DR2 element of the HD-PPRE. *Top*, sequences of the wild-type HD-PPRE and of the oligonucleotides M1, M2, M3 and M4 containing mutations in consensus hexameric binding half-sites I, II, III and IV (*bold lettering*) of the HD-PPRE. *Bold underlined lettering*, sequences of mutant sites I, II, III and IV. *Bottom*, *in vitro* synthesized CARβ and RXRα were incubated with radiolabeled double-stranded wild-type and mutant HD-PPRE probes. Lysate volumes were kept constant by the addition of unprogrammed lysate. EMSA was performed as described under "Materials and Methods". The position of the CARβ/RXRα complex is indicated.

III and IV (the 3' DR1 element) of the HD-PPRE for binding (Fig. 4-3) (Zhang *et al.*, 1993; Winrow [illegible] *al.*, 1998). Disruption of half-sites II and III (comprising the DR2 element) abrogated CARβ/RXRα binding, while half-sites I and IV were dispensable for CARβ/RXRα binding (Fig. 5-3). These results show that CARβ/RXRα and PPARα/RXRα overlap in their binding to the HD-PPRE at half-site III.

5.2.3 CARβ Competes with PPARα for Binding to the HD-PPRE

To determine the influence of CARβ on PPARα/RXRα binding to the HD-PPRE, EMSA was performed using radiolabeled HD-PPRE, constant amounts of PPARα and RXRα, and increasing amounts of CARβ. Increasing the amount of CARβ reduced the binding of PPARα/RXRα on the HD-PPRE and led to a concomitant increase in CARβ/RXRα binding (Fig. 5-4). This result suggests a competition between CARβ and PPARα for limiting amounts of the heterodimerization partner, RXRα. Moreover, the data are suggestive of a dynamic balance between CARβ/RXRα and PPARα/RXRα for binding to the HD-PPRE.

5.2.4 CARβ Differentially Affects PPARα/RXRα-Mediated Transactivation from the HD- and AOx-PPREs

To investigate the effects of CARβ on transcriptional regulation mediated by PPARα/RXRα, we carried out transient transfection assays using luciferase reporter plasmids containing the HD-PPRE (pHD(X3)*luc*) and AOx-PPRE (pAOx(X2)*luc*), along with expression plasmids for PPARα, RXRα and CARβ. Cotransfection of PPARα and

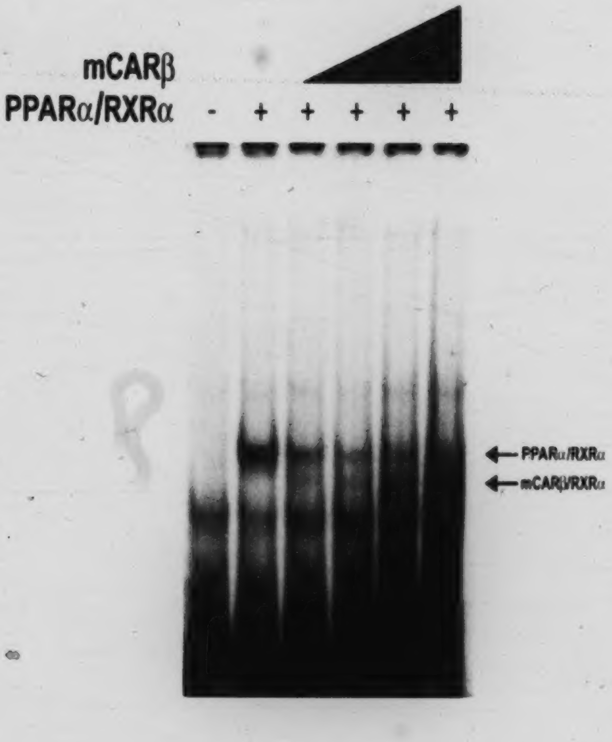


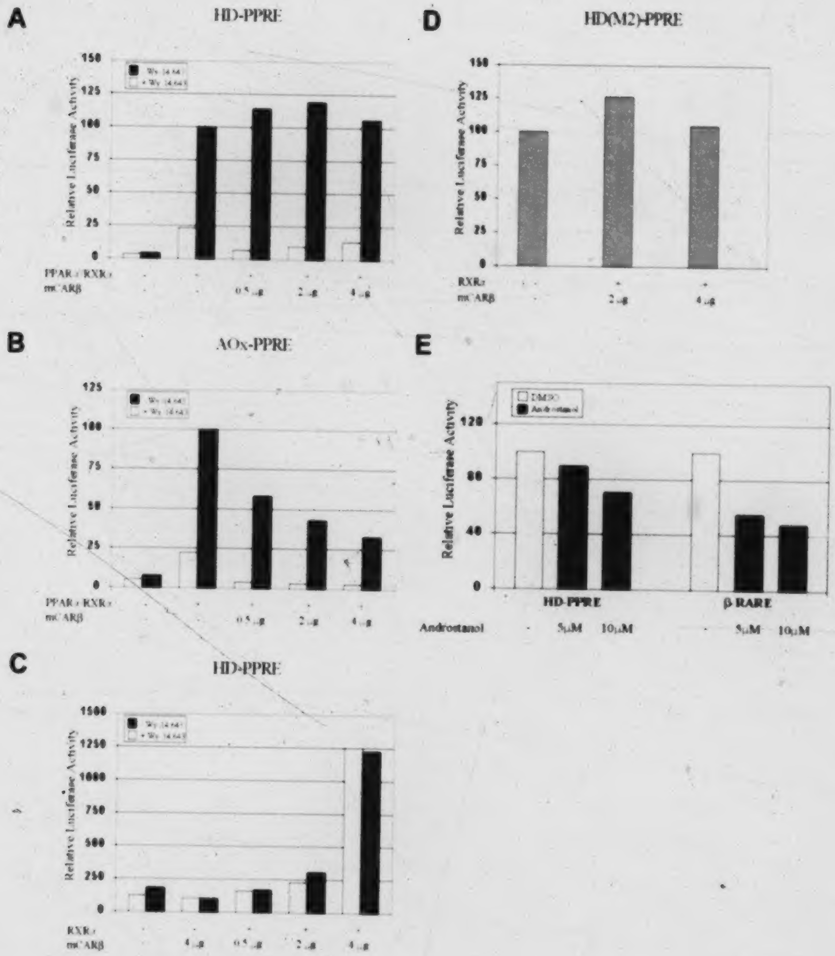
Figure 5-4. CARβ competes with PPARα for RXRα in heterodimer formation on the HD-PPRE. *In vitro* translated RXRα (1 μL) and limiting amounts of PPARα (0.2 μL) were incubated with increasing amounts of *in vitro* translated CARβ (4, 8, 12 and 16 μL), as shown. Lysate volumes were kept constant by the addition of unprogrammed lysate as appropriate. Radiolabeled HD-PPRE was added to the binding reactions following a 5 min pre-incubation, and reactions were kept at 25°C for an additional 25 min before electrophoresis.

RXRα expression plasmids with the HD-PPRE reporter plasmid led to an approximately 6-fold induction in the levels of transcription over basal levels in the absence of the peroxisome proliferator Wy-14,643, and to an approximately 20-fold induction in the levels of transcription in the presence of proliferator (Fig. 5-5, A). Increasing amounts of the expression plasmid for CARβ did not significantly affect PPARα/RXRα-mediated transcription in the presence of proliferator and modestly reduced transcription levels in its absence (Fig. 5-5, A). Interestingly, increasing amounts of CARβ expression plasmid antagonized PPARα/RXRα-mediated transactivation from the AOx-PPRE in a dose-dependent manner, in the presence and absence of Wy-14,643 (Fig. 5-5, B). These data illustrate that CARβ differentially affects PPARα/RXRα-mediated transcription from the HD-PPRE and AOx-PPREs.

5.2.5 CARβ/RXRα Potentiates Transactivation from the HD-PPRE

Since we observed that CARβ/RXRα heterodimers could bind the HD-PPRE *in vitro*, we sought to determine whether the HD-PPRE is a specific target for CARβ/RXRα-mediated transcription *in vivo*. Transient transfections of BSC40 cells were carried out with the pHD(X3)*luc* reporter plasmid in the absence or presence of expression plasmid for RXRα and with varying amounts of CARβ expression plasmid. We observed that CARβ in the presence of coexpressed RXRα could potentiate transcription from the HD-PPRE in a dose-dependent manner, and with equal competence in both the absence and presence of the peroxisome proliferator, Wy-14,643 (Fig. 5-5, C). In the presence of exogenously expressed RXRα, transcription from the HD-PPRE was induced

Figure 5-5. Transactivation by PPARα/RXRα from the HD- and AOx-PPREs is differentially modulated by CARβ. BSC40 monolayer cells were transfected with 5 μg of the luciferase reporter pHD(X3)*luc* (A) or pAOx(X2)*luc* (B) and expression plasmids for PPARα (2 μg), RXRα (2 μg) and CARβ (0.5-4 μg) in the absence or presence of 0.1 mM Wy-14,643. Plasmid dosage was normalized by the addition of empty expression vector. Cells were harvested 48 h post-transfection and luciferase activity quantitated. Transfections were carried out in duplicate, and the values reported represent the average of three independent experiments. Values from independent experiments did not vary by more than 15%. (C) Transfections of BSC40 cells were carried out with 5 μg of the reporter plasmid pHD(X3)*luc* in the absence or presence of RXRα expression plasmid (2 μg) and varying amounts of CARβ expression plasmid (0.5-4 μg), and in the absence or presence of the peroxisome proliferator, Wy-14,643 (0.1 mM). Transfections were carried out in duplicate and represent the average of three independent experiments. Values from independent transfections did not vary by more than 15%. (D) Half-site II of the HD-PPRE is required for transactivation by CARβ/RXRα. BSC40 cells were transfected with 5 μg of the reporter plasmid pM2(X3)*luc*, which harbors a mutation in the second TGACCT-like half-site of the HD-PPRE, and with expression plasmids for RXRα (2 μg) and CARβ (2 μg or 4 μg), as indicated. The values shown represent the averages of three independent transfections carried out in duplicate. Values from independent transfections did not vary by more than 15%. (E) The CARβ ligand, 5α-androstan-3α-ol, reduces transactivation from the HD-PPRE by CARβ/RXRα. Transient transfections were performed in BSC40 cells with expression plasmids for PPARα (2 μg), RXRα (2 μg), and CARβ (4 μg) and with 5 μg of the luciferase reporter plasmids pHD(X3)*luc* or pβRARE(X2)-TK-*luc*, in the absence or presence of 5α-androstan-3α-ol (5 and 10 μM). The values reported represent the averages of three independent transfections done in duplicate. Values from independent transfections did not vary more than 15%.


A
HD-PPRE
Relative Luciferase Activity
150
125
100
75
50
25
0
Wy 14,643
+ Wy 14,643
PPARα/RXRα
mCARβ
0.5 μg
2 μg
4 μg
B
AOx-PPRE
125
100
75
50
25
0
C
HD-PPRE
1500
1250
1000
750
500
250
0
RXRα
4 μg
D
HD(M2)-PPRE
RXRα
mCARβ
2 μg
4 μg
E
DMSO
Androstanol
120
80
40
0
HD-PPRE
β-RARE
Androstanol
5μM
10μM

approximately 12-fold over basal levels with 4 μg of CARβ expression plasmid. This potentiation of transcription by CARβ/RXRα was abrogated when a reporter plasmid harboring a mutation at half-site II of the HD-PPRE (pM2(X3)*luc*) was used in transfection (Fig. 5-5, D), in agreement with *in vitro* results demonstrating that the integrity of half-site II of the HD-PPRE is required for CARβ/RXRα binding (see Fig. 5-3).

5.2.6 The CARβ Ligand 5α-Androstan-3α-ol Reduces Transactivation from the HD-PPRE by CARβ/RXRα

The steroid androstanol metabolite, 5α-androstan-3α-ol, has recently been shown to serve as a ligand for the CARβ receptor (Forman *et al.*, 1998). Interestingly, this ligand acts to reduce transcriptional activation from a β2-RARE by the CARβ receptor (Forman *et al.*, 1998). We investigated whether 5α-androstan-3α-ol would also inhibit transactivation from the HD-PPRE by CARβ/RXRα. In transient transfections performed in the presence of expression plasmids for CARβ and RXRα, addition of 5α-androstan-3α-ol led to an approximately 50% reduction in transcriptional activity of a luciferase reporter construct containing two copies of the β2-RARE (pβRARE(X2)-TK-*luc*) and to an approximately 30% reduction in transcriptional activity of a reporter construct containing three copies of the HD-PPRE (pHD(X3)*luc*) (Fig. 5-5, E). These results suggest that in BSC40 cells, the CARβ ligand retains moderate transcriptional inhibitory effects, with a reporter plasmid containing two copies of the βRARE showing slightly greater transcriptional inhibition than a reporter containing three copies of the HD-PPRE.

5.3 Discussion

Because of the abundant expression of the nuclear hormone receptor CARβ in liver and its functional interaction with RXRα, we investigated whether CARβ had a role in the peroxisomal β-oxidation of fatty acids through regulation of transcription of the HD and AOx genes via their PPREs. We have demonstrated that the HD-PPRE is a target for CARβ/RXRα heterodimers and that CARβ/RXRα stimulates transcription from the HD-PPRE. This increase in transcriptional activity is approximately the same in either the absence or presence of the peroxisome proliferator, Wy-14,643, affirming the ligand-independent transactivity that has previously been demonstrated for CARβ (Baes *et al.*, 1994).

The presence of CARβ does not affect transactivation from the HD-PPRE by PPARα/RXRα. In contrast, CARβ serves to antagonize PPARα/RXRα-mediated transcriptional induction from the AOx-PPRE. We attribute the effects of CARβ on transcription from the HD- and AOx-PPREs in the presence of PPARα to the availability of RXRα required for heterodimerization with both CARβ and PPARα. Since CARβ/RXRα heterodimers form a complex on the HD-PPRE, we suggest that CARβ sequesters RXRα away from PPARα to form a heterodimeric CARβ/RXRα complex that is less transcriptionally robust than the PPARα/RXRα complex on the HD-PPRE. In the case of the AOx-PPRE, which does not bind CARβ/RXRα, sequestration of RXRα away from PPARα by CARβ would result in decreased transcriptional activity, likely as a result of the formation of nonbinding, transcriptionally inactive CARβ/RXRα heterodimers, concomitant with a reduction in the number of transcriptionally active PPARα/RXRα heterodimers.

The involvement of cofactors ~~such~~ as SRC-1, p300, NCoR and SMRT-1 in transcriptional regulation by nuclear hormone receptors is well established (Chen and Evans, 1995; DiRenzo *et al.*, 1997; Dowell *et al.*, 1997,1999). The binding of the ligand 5α-androstan-3α-ol to CARβ has been suggested to result in the dissociation of SRC-1 from CARβ, thereby bringing about transcriptional deactivation (Forman *et al.*, 1998; Kliewer *et al.*, 1999). Addition of 5α-androstan-3α-ol in transient transfections did result in transcriptional deactivation by CARβ/RXRα from both the β-RARE and the HD-PPRE; however, the extent of deactivation was less than what has previously been reported. The observed decrease in deactivation could stem from endogenous levels of PPARα and RXRα in BSC40 cells, which may overcome androstanol deactivation by promoting transcriptional induction from the HD-PPRE via PPARα/RXRα heterodimers. PPARα/RXRα heterodimers bind weakly to the β2-RARE (Kliewer *et al.*, 1992b), and therefore endogenous cellular levels of PPARα and RXRα would probably be precluded from potentiating transcription from the β2-RARE, allowing for the observed deactivation by CARβ in the presence of androstanol. Surprisingly, we observed only a moderate reduction of ligand-independent activity by CARβ/RXRα on the β2-RARE as compared to previously published results (Forman *et al.*, 1998). These differences could be attributed to inherent differences in the cell lines used, including the relative levels of endogenous nuclear hormone receptors, corepressors and coactivators. These and other cell-specific factors could influence the observed differences in ligand-dependent deactivation.

The HD-PPRE is a complex response element composed of four hexameric DRs arranged as two tandem DR1 arrays separated by two nucleotides, thereby forming a DR2. This complex arrangement permits the interaction of a variety of nuclear hormone receptors on the HD-PPRE (Marcus *et al.*, 1993; Miyata *et al.*, 1993; Winrow *et al.*, 1994, 1998), leading to a complex model of transcriptional regulation from this response element. CARβ/RXRα heterodimers bind to half-sites on the HD-PPRE that partly overlap with half-sites recognized by PPARα/RXRα heterodimers. Half-sites II and III of the HD-PPRE, which form a DR2, are required for CARβ/RXRα binding, while PPARα/RXRα binds to sites III and IV. CARβ/RXRα-mediated transactivation is eliminated when half-site II or III is mutated. It has been previously reported that half-site III of the HD-PPRE is occupied by RXRα, while the more distal half-site (IV) is occupied by PPARα (Chu *et al.*, 1995). Considering this, and the fact that CARβ/RXRα heterodimers have been shown to bind β2-RARE with DR2 spacing (Choi *et al.*, 1997), it is not surprising that CARβ would likely occupy half-site II, which matches perfectly the consensus hexameric half-site sequence. Furthermore, the intricate combinations of various nuclear hormone receptors that can modulate the transcriptional response from the HD-PPRE converge on the second hexameric half-site (Winrow *et al.*, 1998; Chapters 3 and 4). Nevertheless, the limitations of our *in vitro* assays do not allow us to distinguish which half-site is specifically occupied by CARβ when bound as a heterodimer with RXRα on the HD-PPRE.

Our findings suggest that CARβ can modulate the transcriptional response of the genes coding for two enzymes of the peroxisomal β-oxidation pathway, AOx and HD.

The peroxisomal β-oxidation pathway is involved preferentially in the metabolism of long- and very long-chain fatty acyl-CoAs. AOx, the first enzyme in the pathway, is rate-limiting. We have shown that CARβ decreases transcriptional activation of the AOx gene while increasing transcriptional activation of the HD gene, which encodes the second enzyme of the peroxisomal β-oxidation pathway. This differential regulation of the genes encoding the first two enzymes of the peroxisomal β-oxidation pathway may represent an adaptive cellular response that primes the pathway to respond rapidly to cellular oxidative demands under physiological conditions where repression of the transcription of the AOx gene is relieved.

5.4 Summary

In summary, we demonstrate that CARβ/RXRα heterodimers play a role in fatty acid homeostasis by regulating the transcription of the genes encoding the first two enzymes of the peroxisomal β-oxidation pathway, acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. We also show that the PPRE of the HD gene is a target for CARβ/RXRα heterodimers. The convergence of the CARβ, PPARα and RXRα signaling pathways underscores the complex and dynamic processes by which various metabolic cues are integrated to elicit the correct transcriptional response leading to control of peroxisomal β-oxidation.

CHAPTER 6

The Short Heterodimer Partner Receptor Differentially Modulates PPARα-Mediated Transcription from Peroxisome Proliferator-Response Elements of the Genes Encoding Acyl-CoA Oxidase and Hydratase-Dehydrogenase

6.1 Abstract

The short heterodimer partner (SHP) receptor is expressed in the liver and has been shown to interact with a number of nuclear hormone receptors including PPARα (Seol *et al.*, 1996; Masuda *et al.*, 1997). In this study, the role of SHP in modulating PPARα-mediated gene transcription from the PPREs of the genes AOx and HD was investigated both *in vitro* and *in vivo*. *In vitro* binding assays using GST-tagged chimeric receptors for PPARα and SHP were used to verify the interaction between PPARα and SHP. This interaction was unaffected by the presence of the peroxisome proliferator, Wy-14,643. SHP is proposed to act as a negative regulator of nuclear hormone receptor activity, and therefore it was not surprising that SHP inhibited transcription by PPARα/RXRα heterodimers from the AOx-PPRE. Surprisingly, SHP potentiated transcription by PPARα/RXRα heterodimers from the HD-PPRE. This is the first demonstration of positive transcriptional activity attributable to SHP. Togther, these results suggest that SHP modulates PPARα/RXRα-mediated transcription in a response element-specific manner.

6.2 Results

6.2.1 SHP Interacts with PPARα and RXRα In Vitro

SHP was initially isolated by its interaction with the ligand binding domain/AF2 domain of PPARα and CARβ (Seol *et al.*, 1996; Johansson *et al.*, 1999). To verify the physical interaction between SHP and PPARα, *in vitro* binding assays were performed using GST-fusion proteins or GST alone with *in vitro* translated, full-length, ^{35}S-methionine-labeled PPARα and SHP receptors. PPARα could interact specifically with GST-SHP, but not GST alone (Fig. 6-1, A, *upper panel*). Approximately 8 to 10 % of the input radiolabeled PPARα bound to GST-SHP (compare lane 3 to lane 1). This interaction was also confirmed in a reciprocal manner in which SHP bound specifically to GST-PPARα but not GST alone (Fig. 6-1, A, *lower panel*). Furthermore, this interaction was unaffected by the presence of 0.1 mM Wy-14,643 or vehicle, as binding efficiencies were the same under both conditions (Fig. 6-2, A). Control reactions verified the interaction between PPARα and RXRα (Fig. 6-2, A, *bottom panel*). We also demonstrated that SHP and RXRα could interact *in vitro* (Fig. 6-2, B *top panel*) and that the binding between SHP and RXRα could be increased in the presence of 1 μM 9-*cis* retinoic acid (Fig. 6-2, B, *top and middle panels*), in agreement with previously published results (Seol *et al.*, 1996). However, 9-*cis* retinoic acid had no effect on the interaction between PPARα and RXRα (Fig. 6-2, B, *bottom panel*). These results indicate that SHP can physically interact with both PPARα and RXRα in the absence or presence of their respective cognate ligands.

6.2.2 SHP Modulates PPARα/RXRα-Mediated Transcription in a Response Element-Specific Manner

Transient transfection assays were used to examine the effects of SHP on PPARα-mediated transcription *in vivo*. Assays included a luciferase reporter gene containing either three copies of the HD-PPRE or two copies of the AOx-PPRE. As shown in Figure 6-3 *A*, cotransfection of PPARα and RXRα led to a 3-fold and 10-fold induction of

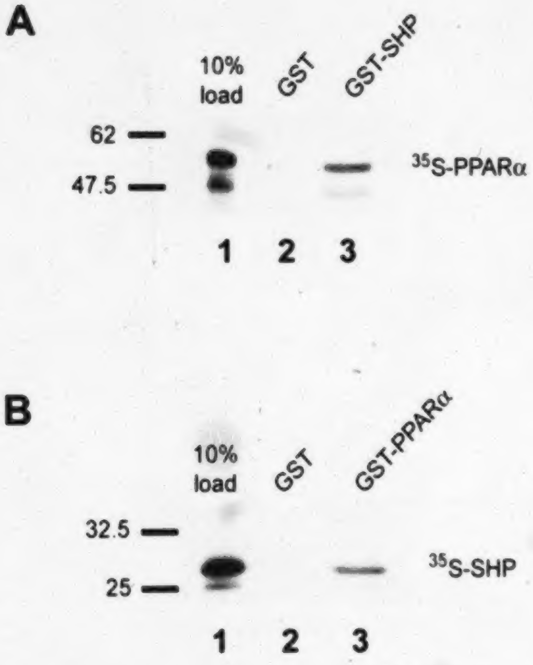
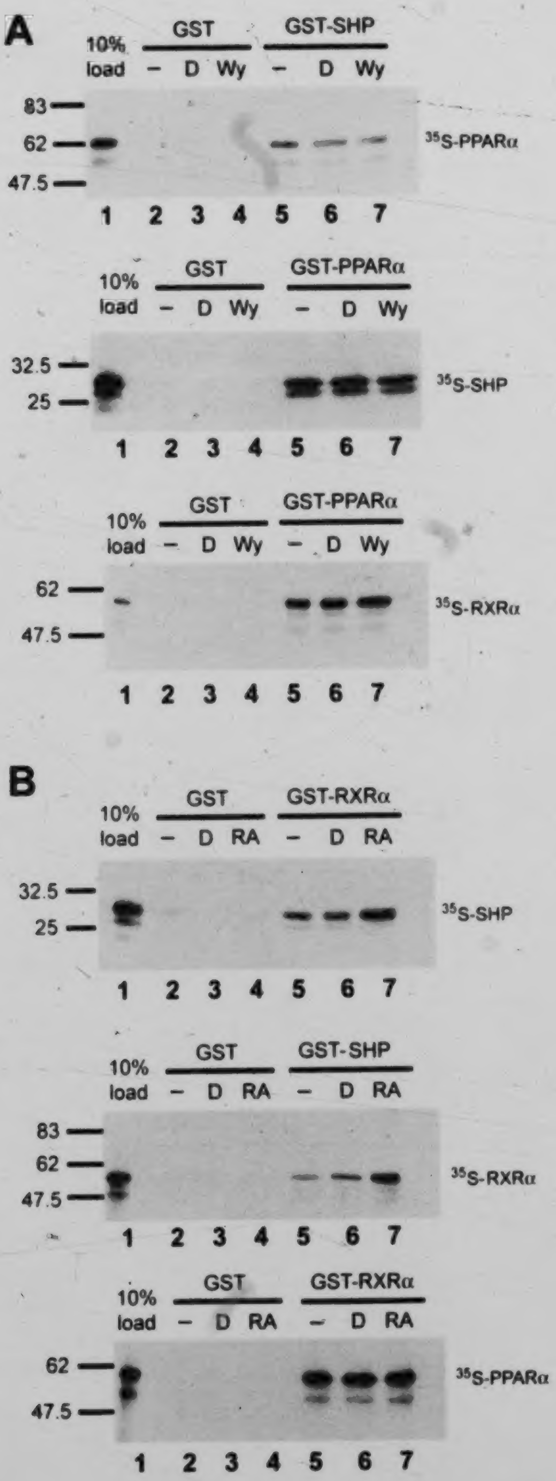

A
10% load
GST
GST-SHP
62
47.5
^{35}S-PPARα
1
2
3
B
10% load
GST
GST-PPARα
32.5
25
^{35}S-SHP
1
2
3

Figure 6-1. PPARα and SHP interact *in vitro*. (A) L-[^{35}S]methionine-labeled full-length PPARα or (B) SHP synthesized *in vitro* were incubated with either GST-SHP (A, lane 3) or GST-PPARα (B, lane 3), respectively, or GST alone (A and B, lane 2), as indicted. Bound radiolabeled protein was analyzed by SDS-PAGE. Lane 1 represents 10% of the labeled material added to each of the binding reactions.

Figure 6-2. Wy-14,643 does not affect the binding efficiency between PPARα and SHP. (A) Combinations of L-[^{35}S]methionine-labeled full-length PPARα or SHP were coincubated with GST-SHP or GST-PPARα, respectively, alone (*top and middle panels*; lanes 2 and 5) or in the presence of 100 μM Wy-14,643 (Wy) (lanes 4 and 7) or an equivalent volume of vehicle (dimethylsulfoxide (D)) (lanes 3 and 6). Bound material was analyzed as in Figure 6-1. ,(B) The interaction of SHP and RXRα *in vitro* is enhanced in the presence of 9-*cis* retinoic acid. Binding reactions with L-[^{35}S]methionine-labeled full-length RXRα and SHP incubated with GST-SHP and GST-RXRα respectively (*top and middle panels*) were analysed by SDS-PAGE. Where indicated, reactions contained 1 μM 9-*cis* retinoic acid (RA) (lanes 4 and 7), or an equivalent volume of vehicle (dimethylsulfoxide (D)) (lanes 3 and 6). Lanes 2 and 5 represent binding reactions in the absence or vehicle or ligand. Control binding reactions were performed with PPARα and RXRα (A and B; *bottom panels*). In all cases, lane 1 represents 10% of each radiolabeled receptor added to the binding reactions.


A
10% load
GST
GST-SHP
– D Wy
– D Wy
83
62
47.5
35S-PPARα
1 2 3 4 5 6 7
10% load
GST
GST-PPARα
– D Wy
– D Wy
32.5
25
35S-SHP
1 2 3 4 5 6 7
10% load
GST
GST-PPARα
– D Wy
– D Wy
62
47.5
35S-RXRα
1 2 3 4 5 6 7
B
10% load
GST
GST-RXRα
– D RA
– D RA
32.5
25
35S-SHP
1 2 3 4 5 6 7
10% load
GST
GST-SHP
– D RA
– D RA
83
62
47.5
35S-RXRα
1 2 3 4 5 6 7
10% load
GST
GST-RXRα
– D RA
– D RA
62
47.5
35S-PPARα
1 2 3 4 5 6 7

transcription from the AOx-PPRE in the absence and presence of the Wy-14,643, respectively. As increasing amounts of expression plasmid for SHP were cotransfected, there was a dose-dependent inhibition of PPARα/RXRα-mediated gene transcription with an approximately 80% reduction in PPARα/RXRα transactivity in the presence of 0.1 mM Wy-14,643. Interestingly, when a reporter plasmid harbouring three copies of the HD-PPRE was used in transient transfections, increasing amounts of expression plasmid for SHP potentiated PPARα/RXRα-mediated gene transcription (Fig. 6-3, B). At the highest amounts of SHP expression plasmid used, ligand-dependent transcription was increased approximately 3-fold. These *in vivo* data illustrates that PPARα/RXRα-mediated gene transcription can be modulated by SHP in a response element-specific manner.

6.3 Discussion

The effect of SHP on individual nuclear hormone receptors is dependent on several factors that may include the intrinsic activity of the nuclear hormone receptor itself, the relative expression levels of SHP, its potential heterodimeric partners, and their relative affinity for one another. Additionally, the presence of agonist or antagonist ligands may induce conformational changes in SHP, enabling it to recruit coactivators or corepressors, and thereby function as a transcriptional activator or repressor, respectively (Johansson *et al.*, 1999). A number of models have been proposed to explain transcriptional repression by SHP. These include inhibiting DNA binding by various receptor heterodimers by competition for the common heterodimeric partner RXRα (Seol *et al.* 1996; Johansson *et al.*, 1999) and modulating the recruitment of coactivators and corepressors (Seol 1996;

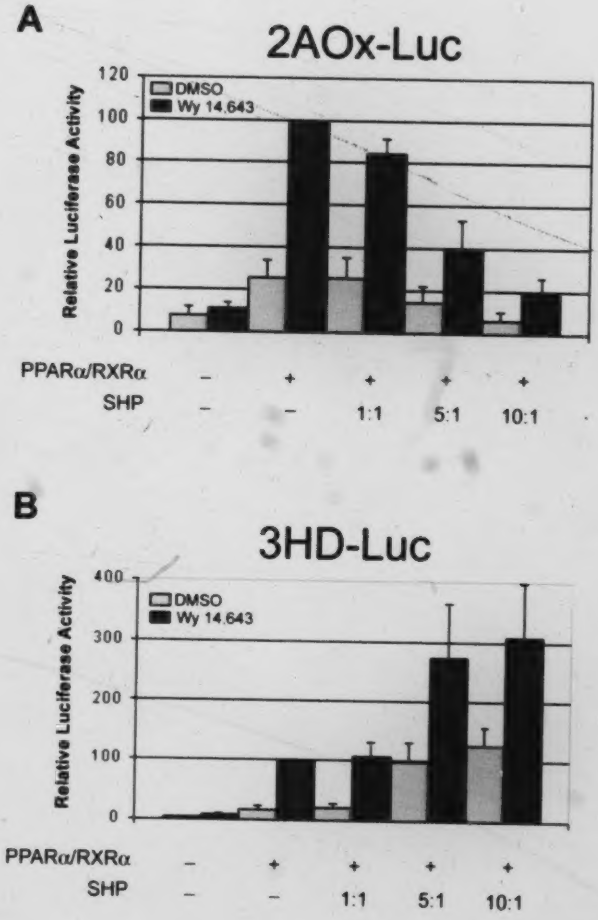


Figure 6-3. SHP differentially modulates PPARα/RXRα-mediated transactivation from the AOx- and HD-PPREs. (A) BSC40 cells were transfected with the reporter plasmid pAOx(X2)*luc* and expression plasmids for PPARα (0.5 μg), RXRα (0.5 μg), and increasing amounts of SHP (1x, 5x, or 10x) relative to the total amount of PPARα and RXRα expression plasmids. (B) Transfections were carried as in (A) but with the pHD(X3)*luc* reporter plasmid. Values represent the average (±SEM) of three separate experiments carried out in duplicate and normalized to the activity with PPARα/RXRα in the presence of Wy-14,643 (taken as 100%).

Johansson *et al.*, 1999; Lee *et al.*, 2000). Recently, it has been demonstrated that SHP contains a putative carboxyl-terminal autonomous repression domain (Seol *et al.*, 1997; Johansson *et al.*, 1999) that functions to repress transactivation by a two-step mechanism. In this model, an initial inhibitory effect occurs from the loss of coactivator binding due to competition by SHP. Next, full inhibition is exerted by the SHP repressor domain by an as yet unknown mechanism (Lee *et al.*, 2000). Since repression has been demonstrated to be a general feature of SHP, this explanation may also account for its repressive effect on PPARα/RXRα-mediated transcription from the AOx-PPRE.

Typically, coactivators bind to the AF2 regions of nuclear receptors via LXXLL motifs. Johansson and colleagues (2000) have recently demonstrated that SHP can bind to estrogen receptors via LXXLL-related motifs. The existence of these similar motifs within the putative ligand binding domain of SHP suggests that SHP may mimic the interaction of some transcriptional coactivators. (Johansson *et al.*, 2000). Therefore, SHP may preclude classical coactivators from binding to ligand-activated nuclear hormone receptors and, in combination with the above cited two-step mechanism of repression, may explain the inhibitory activity of SHP. Conversely, given that SHP contains LXXLL-like motifs, SHP could also function as a novel coactivator molecule. This would partially explain SHP transactivity on PPARα/RXRα-mediated transcription from the HD-PPRE. Therefore, it appears that SHP shares the physical features of both a nuclear hormone receptor and a novel "coactivator-like repressor" that binds to the AF2 domain of nuclear hormone receptors. The dual modality of SHP may also result from allosteric effects exerted by the response element. The PPREs can induce a conformational change in

PPARα/RXRα heterodimers that enable the heterodimer to either bind strong, positively acting coactivators or bind SHP alone or with cellular cofactors to potentiate transactivation. Alternatively, spatial constraints conferred by response elements may cause PPARα and RXRα to bind SHP, precluding the binding of coactivators causing transrepression.

In gel electromobility shift assays, SHP failed to bind either the AOx- or HD-PPRE alone or in combination with PPARα or RXRα. SHP lacks a conventional DNA binding domain, likely accounting for its inability to bind to various response elements (Seol *et al.*, 1996). However, it may have the potential to do so *in vivo*. DAX-1, another orphan nuclear receptor that lacks a DNA binding domain, has been reported to bind a retinoic acid response element using a novel amino-terminal domain. This raises the possibility that SHP may do so similarly. Additionally, the lack of binding to PPREs *in vitro* also suggests that complex formation *in vivo* may be dependent on additional cellular cofactors that stabilize such a complex. Binding assays containing mammalian cell extracts expressing SHP, PPARα, and RXRα may provide insight into the formation of a SHP-specific complex *in vivo*.

6.4 Summary

Our results demonstrate that SHP can differentially modulate PPARα/RXRα-mediated gene transcription from the AOx- and HD-PPREs. Given our limited analysis, a number of mechanisms can be proposed to account for the differential activity of SHP, including the role of coactivators and corepressors, as well as allosteric effects from the response elements themselves. Further studies are required to delineate the exact mechanism by which SHP acts as a transactivator or transrepressor. The potential of SHP to silence or redirect ligand signaling events presents a novel and unique mechanism of crosstalk between nuclear hormone receptors and places SHP structurally and functionally between nuclear receptors and their associated transcriptional cofactors.

CHAPTER 7

Subtype- and Response Element-Dependant Differences in Transactivation by Peroxisome Proliferator-Activated Receptors α and γ[§]

[§] A copy of this chapter has been published. Kassam, A., Hunter, J., Rachubinski, R. A., and Capone, J. P. *Mol. Cell. Endocrinol.* 141:153-162 (1998). Used with permission from Elsevier Science.

7.1 Abstract

Evidence from transfection and DNA binding studies suggest that PPAR activity may be subtype- and PPRE-dependent (Marcus *et al.*, 1993; Juge-Aubry *et al.*, 1997). However, direct examination of PPAR subtype properties in mammalian cells is complicated by the presence of multiple endogenous nuclear receptors, cofactors and potentially activating ligands that may directly, or indirectly, modulate PPAR activity and DNA binding. Yeast offer a number of advantages, since they are devoid of endogenous nuclear receptors and nuclear receptor-specific auxiliary cofactors (Butt and Walfish, 1996). Furthermore, it has been previously shown that PPARα/RXRα activates transcription in a PPRE-dependent, ligand-independent manner in the yeast *Saccharomyces cerevisiae* (Marcus *et al.*, 1995). To investigate potential subtype- and response element-dependent differences in transcriptional activation by PPARs, plasmids containing cDNAs for PPARα and PPARγ2, along with RXRα, were expressed in *S. cerevisiae* in order to compare their ability to activate transcription of reporter genes containing a PPRE from either the rat acyl-CoA oxidase (AOx) or enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) gene. PPARγ2 and RXRα, when coexpressed from low copy vectors, potently and synergistically activated transcription from the AOx-PPRE reporter gene, but only weakly stimulated transcription from the HD-PPRE reporter gene. This response element preference, which was also observed in mammalian cells, could not be attributed to differences in binding affinity of PPARγ2/RXRα heterodimers to these elements *in vitro*. Interestingly, PPARγ2 expressed from a high copy vector was able to strongly activate transcription from the HD-PPRE reporter gene, even in the absence of

exogenous RXRα. In comparison to the findings with PPARγ2, the HD-PPRE served as a significantly more robust response element for PPARα as compared to the AOx-PPRE. PPRE-dependent transcriptional activation by PPARα correlated with binding efficiencies of PPARα/RXRα to the response element. These findings demonstrate that the transactivation potential of PPAR subtypes can be differentially modulated by distinct PPREs.

7.2 Results

7.2.1 Properties of PPARα and PPARγ2 Expressed in Yeast

To examine PPAR subtype and PPRE-dependent specificities in yeast, we expressed PPARα, PPARγ2, and RXRα, from low copy *CEN* vectors and used *lacZ* reporter genes containing a single copy of either the HD- or AOx-PPRE. As summarized in Table 7-1, transformation of yeast with the HD-PPRE reporter gene and either PPARα or RXRα expression plasmids had little effect on reporter gene activity. However, coexpression of both receptors led to a greater than100-fold induction in activity relative to the reporter gene transformed alone. The level of induction with PPARα/RXRα was observed to be only 4-fold with the AOx-PPRE reporter. In terms of specific activity, PPARα/RXRα generated a 2 to 3-fold greater specific activity on the HD-PPRE as compared to the AOx-PPRE (2.8×10^2 vs 1.2×10^2 U/mg protein, respectively). The larger difference in the relative fold induction compared to specific activity is due to the higher basal activity from the AOx-PPRE reporter gene. Similar results were obtained when receptors were expressed from high copy plasmids (Table 7-1, B). PPARα/RXRα

Table 7-1. (A) *S. cerevisiae* harbouring the β-galactosidase reporter genes and low copy plasmids expressing PPARα, PPARγ2, and/or RXRα, as indicated, were assayed for β-galactosidase activity. (B) *Following page*, transformation were carried out as in (A) using high copy plasmids. Units are given as the $\Delta A_{420} \times 10^3$/min. The values shown are the averages (±SEM) of at least three independent transformants which were assayed in duplicate.

A *Transcriptional Activation by PPARα and PPARγ2 Expressed in Yeast from Low Copy Vectors*

Reporter	RXRα	PPARα	PPARγ2	β-galactosidase (U/mg protein)	Induction Ratio
1AOxΔL1	-	-	-	32 ± 5.6	1
	+	-	-	49 ± 6.1	1.5
	-	+	-	24 ± 3.2	0.8
	+	+	-	124 ± 12.4	3.9
	-	-	+	53 ± 6.5	1.6
	+	-	+	1206 ± 120	38
1HDΔL1	-	-	-	2.4 ± 0.3	1
	+	-	-	4.4 ± 1.2	1.9
	-	+	-	2 ± 0.07	0.8
	+	+	-	277 ± 17	118
	-	-	+	17 ± 0.4	7.3
	+	-	+	33 ± 4	14

B *Transcriptional Activation by PPARα and PPARγ2 Expressed in Yeast from High Copy Vectors*

Reporter	RXRα	PPARα	PPARγ2	β-galactosidase (U/mg protein)	Induction Ratio
1AOxΔL1	-	-	-	48 ± 3.4	1
	+	-	-	89 ± 16.1	1.9
	-	+	-	59 ± 7.1	1.2
	+	+	-	810 ± 93	17
	-	-	+	92 ± 6.5	1.9
	+	-	+	885 ± 70	18.4
1HDΔL1	-	-	-	8.5 ± 0.8	1
	+	-	-	53 ± 5.2	6.1
	-	+	-	47 ± 3.5	5.5
	+	+	-	2772 ± 220	326
	-	-	+	610 ± 60	72
	+	-	+	1060 ± 84	123

induced expression of the AOx-PPRE reporter gene 17-fold and of the HD-PPRE reporter gene greater than 300-fold (specific activity of 8×10^2 vs 2.7×10^3 U/mg protein, respectively). Therefore, while overall levels of activity were significantly higher when using high copy vectors, the response element preference of PPARα was maintained.

In parallel experiments, PPARγ2 and RXRα individually had no effect on the activity of the AOx-PPRE reporter gene, whereas cotransformation led to a synergistic 38-fold activation (Table 7-1). Thus PPARγ2, like PPARα, is a constitutive transcriptional activator in yeast. The absolute level of activity with PPARγ2 on the AOx-PPRE was an order of magnitude greater than that observed with PPARα (1.2×10^3 vs 1.2×10^2 U/mg protein, respectively). In contrast, PPARγ2/RXRα coexpression led to only a modest induction of the HD-PPRE reporter gene. Therefore, compared to the results with PPARα, the AOx-PPRE serves as a much more efficient response element than does the HD-PPRE for PPARγ2.

Activation of the AOx-PPRE reporter gene by PPARγ2/RXRα expressed from high copy vectors was similar to that observed when low copy expression vectors were used (885 vs 1206 U/mg protein, respectively). However, coexpression of PPARγ2 and RXRα from high copy vectors led to greatly increased activity over those obtained with low copy vectors (1060 vs 33 U/mg protein, respectively). These findings indicate that PPARγ2/RXRα, when expressed from low copy vectors, was limiting for activation from the AOx-PPRE reporter gene. Surprisingly, PPARγ2 was able to strongly induce expression of the HD-PPRE reporter gene in the absence of coexpressed RXRα (Table 7-1). This was particularly evident when PPARγ2 was expressed on a high copy vector (72-

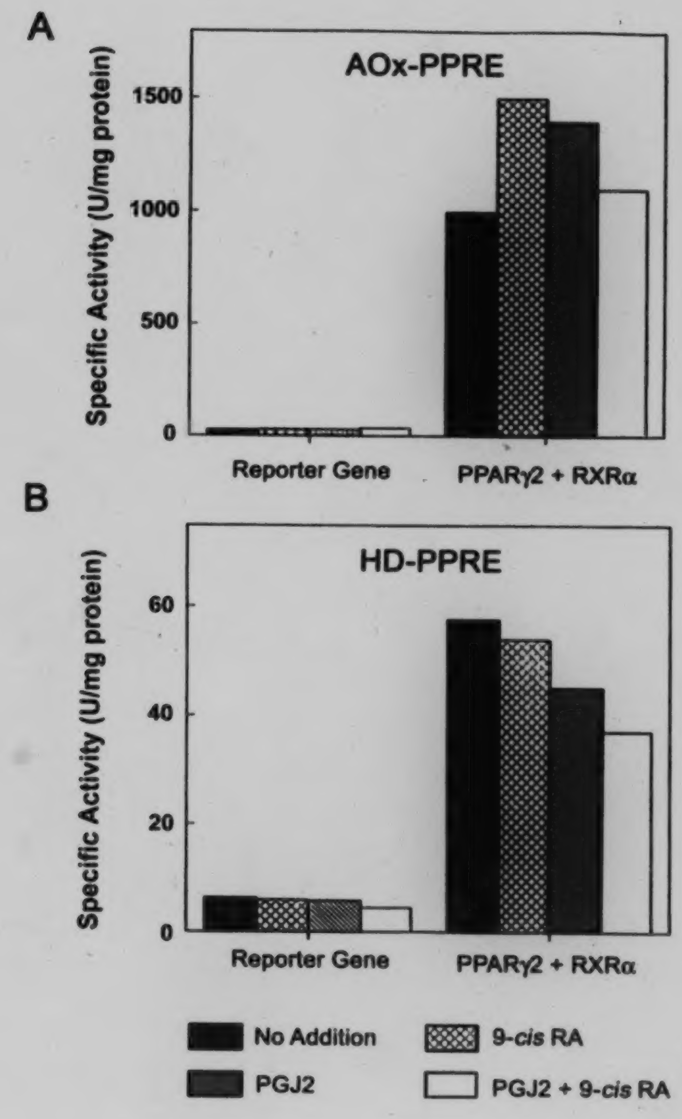
fold induction over basal levels). RXRα-independent activity of PPARγ2 was subtype and response-element-specific, since it was not observed with either the AOx-PPRE reporter gene or PPARα. However, cooperative transactivation between PPARγ2 and RXRα on the HD-PPRE was weak, as coexpression only modestly stimulated activity (1.5-fold) over that observed with PPARγ2 alone.

Overall, these findings demonstrate that both PPARα and PPARγ2 are constitutive activators in yeast but that their respective activities can be differentially modulated by the nature of the target PPREs, availability of the heterodimerization partner RXRα, and levels of expression of the respective PPAR subtypes.

7.2.2 Response of PPARγ2 in Yeast to Exogenously Added Ligand

It has been previously demonstrated that addition of potent exogenous activators of PPARα, such as Wy-14,643 or ciprofibrate, does not further potentiate the activity of PPARα in yeast, irrespective of the target PPRE or the presence of the RXRα-selective ligand, 9-*cis* retinoic acid (Marcus *et al.*, 1995; Henry *et al.*, 1995). The yeast expression assays were extended to determine if PPARγ2 activation could be modulated by its high-affinity ligand, the prostaglandin metabolite 15d-PGJ2. As shown in Figure 7-1, addition of 15d-PGJ2 did not significantly increase transactivation by PPARγ2 when either the HD-PPRE-*lacZ* reporter or the AOx-PPRE-*lacZ* reporter was used. Similarly, addition of 9-*cis* retinoic acid alone and in combination with 15d-PGJ2 also failed to activate PPARγ2. This lack of robust response to potent ligands in yeast is not uncommon and may be due to poor uptake of ligand or lack of coregulatory factors for full activation (Henry *et al.*,

Figure 7-1. PPARγ2/RXRα-mediated transactivation in yeast is not potentiated by the presence of ligand. Yeast transformed with 1AOxΔL1 (A) or 1HDΔL1 (B) reporter genes and low-copy expression plasmids for PPARγ2 and RXRα (or the corresponding empty vectors) were incubated with 15d-PGJ2 and/or 9-*cis* retinoic acid (RA), as indicated, and assayed for β-galactosidase activity. The values shown are averages from two independent assays done in duplicate. Values within individual experiments did not vary by more than 15%.


A
AOx-PPRE
Specific Activity (U/mg protein)
0
500
1000
1500
Reporter Gene
PPARγ2 + RXRα
B
HD-PPRE
Specific Activity (U/mg protein)
0
20
40
60
Reporter Gene
PPARγ2 + RXRα
No Addition
9-*cis* RA
PGJ2
PGJ2 + 9-*cis* RA

1995; Butt and Walfish, 1996).

7.2.3 Comparison of Binding Properties Between PPARα and PPARγ2 on the AOx- and HD-PPREs

EMSA was carried out to determine if the response element- and PPAR subtype-dependent differences in transactivation reflected differences in protein/DNA binding affinities of the PPARα/RXRα and PPARγ2/RXRα heterodimers. Yeast extracts prepared from cells expressing PPARα or PPARγ2 alone, or in combination with RXRα, were incubated with labeled AOx- or HD-PPRE oligonucleotide probes. PPARγ2 bound cooperatively with RXRα, and with similar efficiencies, to the AOx- and HD-PPREs (Fig. 7-2, lane D). PPARγ2 was unable to bind to either PPRE in the absence of RXRα (lane C). PPARα/RXRα also bound to both elements; however, binding appeared to be more efficient on the HD-PPRE (lane F). The PPARγ2/RXRα complex exhibited retarded mobility compared to the PPARα/RXRα complex, likely due to the larger size of PPARγ2. PPARγ2/RXRα apparently bound more avidly that PPARα/RXRα to both PPREs (Fig. 7-2, compare lanes D and F, respectively).

EMSA was also carried out with *in vitro* translated receptors to quantitate the relative binding efficiencies of the PPAR subtypes to the AOx- and HD-PPREs. The specific activities of the PPRE probes did not differ by more than 10%, and PPARα and PPARγ2 receptor molecules were equalized in the binding reactions. PPARα/RXRα bound to the HD-PPRE with a 2 to 3-fold greater efficiency as compared to the AOx-PPRE (Fig. 7-3, A, *upper panel*; compare lanes 4-6 with lanes 10-12). PPARγ2/RXRα

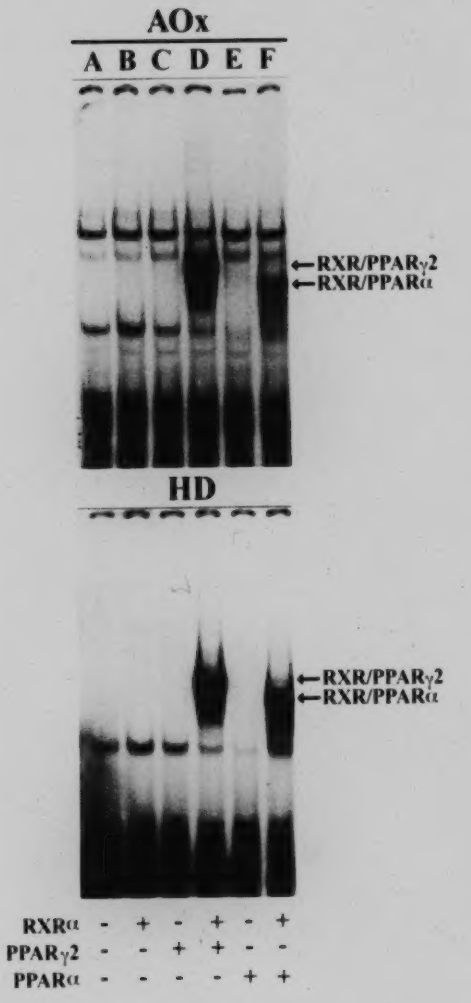
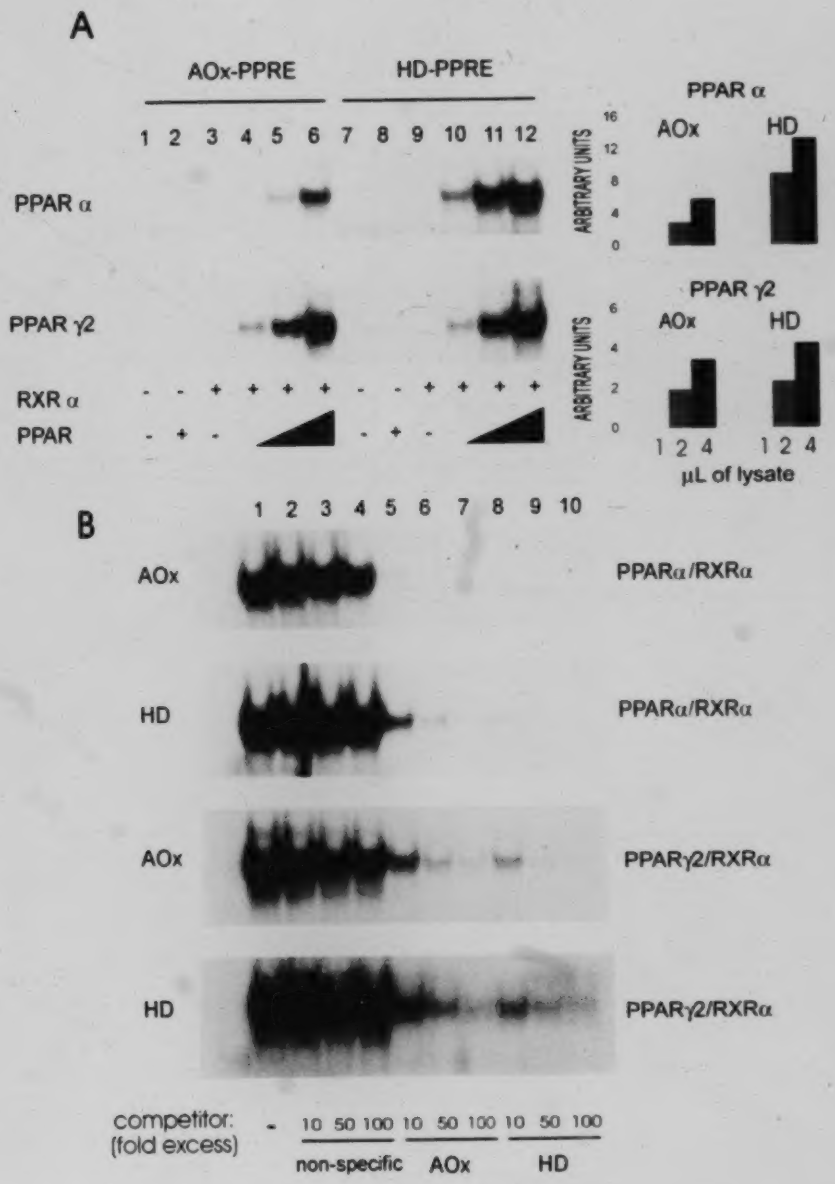


Figure 7-2. PPARγ2 expressed in yeast binds cooperatively with RXRα to the AOx- and HD-PPREs. Electromobility shift assays were performed with extracts prepared from yeast individually expressing PPARα, PPARγ2, or RXRα; or coexpressing PPARα/RXRα or PPARγ2/RXRα, with radiolabeled oligonucleotide probes corresponding to the AOx-PPRE (*upper panel*) or the HD-PPRE (*lower panel*). Additions are indicated at the bottom of the figure. The positions of the PPARγ2/RXRα and PPARα/RXRα protein/DNA complexes are indicated by the arrows. The first lane in each panel represents a control in which the respective probe was incubated with extract prepared from yeast transformed with empty vectors.

Figure 7-3.* Comparison of PPARα/RXRα and PPARγ2/RXRα DNA-binding activities on the HD- and AOx-PPREs. (A) A constant amount of *in vitro* translated RXRα was incubated with increasing amounts of PPARα (upper panel) or PPARγ2 (lower panel) (1:1, 1:2, and 1:4 molar ratios, respectively) with either labeled AOx-PPRE probe or HD-PPRE probe, as indicated, and EMSA was performed. Lanes 1 and 7 represent binding reactions carried out with unprogrammed lysates. Quantitation of PPAR/RXRα protein/DNA complexes, as determined by phosphorimager analysis, is shown on the right. The values given are arbitrary units normalized to the amount of the respective PPAR/RXRα complex formed on the AOx-PPRE (at a PPAR/RXRα molar ratio of 1:1), which was taken as one. (B) Competition analysis of PPAR/RXRα protein/DNA complexes. Labeled AOx- and HD-PPRE oligonucleotides were incubated with PPARα/RXRα or PPARγ/RXRα (1:1 molar ratio) in the absence of competitor DNA (lane 1) or the presence of 10-, 50-, or 100-fold molar excess of unlabeled nonspecific DNA (lanes 2-4), AOx-PPRE DNA (lanes 5-7), or HD-PPRE DNA (lanes 8-10), as indicated. Protein/DNA complexes were resolved by EMSA and quantitated as above.

* *The data for this figure were contributed by John Hunter.*


A
AOx-PPRE
HD-PPRE
1 2 3 4 5 6 7 8 9 10 11 12
PPAR α
PPAR γ2
RXR α
PPAR
- - + + + + - - + + + +
- + -
- + -
PPAR α
AOx
HD
ARBITRARY UNITS
16
12
8
4
0
PPAR γ2
AOx
HD
ARBITRARY UNITS
6
4
2
0
1 2 4
1 2 4
µL of lysate
B
1 2 3 4 5 6 7 8 9 10
AOx
HD
AOx
HD
PPARα/RXRα
PPARα/RXRα
PPARγ2/RXRα
PPARγ2/RXRα
competitor:
(fold excess)
-
10 50 100 10 50 100 10 50 100
non-specific
AOx
HD

bound modestly more efficiently (approximately 25%) to the HD-PPRE than to the AOx-PPRE (Fig. 7-3, A, *lower panel*; compare lanes 4-6 with lanes 10-12). Finally, PPARγ2/RXRα bound more efficiently than PPARα/RXRα to both the AOx- and HD-PPREs (Fig. 7-3; compare lower panel to upper panel).

Competition experiments were performed with increasing concentrations of unlabeled nonspecific and specific double-stranded oligonucleotides to further investigate protein/DNA complex stability (Fig. 7-3, B). HD-PPRE DNA was a more effective competitor than AOx-PPRE DNA, irrespective of the labeled probe used or whether the protein/DNA complexes were generated with PPARα/RXRα or PPARγ2/RXRα (lanes 5-7). In general, 2 to 5 times more unlabeled AOx competitor DNA than HD competitor DNA was required to achieve the same degree of inhibition. Moreover, complexes formed on the AOx-PPRE were more sensitive to disruption by nonspecific DNA, suggesting that complexes formed on the AOx-PPRE were less stable (Fig. 7-3, B, compare lanes 1-4).

7.2.4 PPARα and PPARγ2 Activity in Mammalian Cells

PPAR activity in mammalian cells was measured by transient transfection assays performed in BSC40 cells. PPARγ2/RXRα coexpression led to a 6-fold induction of activity of pAOx(X2)GL as compared to control transfections containing only the pAOx(X2)GL reporter plasmid (Fig. 7-4, A). Activity of pAOx(X2)GL was further augmented by the addition of the PPARγ-specific ligand, 15d-PGJ2, resulting in a 3-fold induction in activity compared to the corresponding control with vehicle alone. The induction in reporter gene activity observed in the absence of transfected receptors is

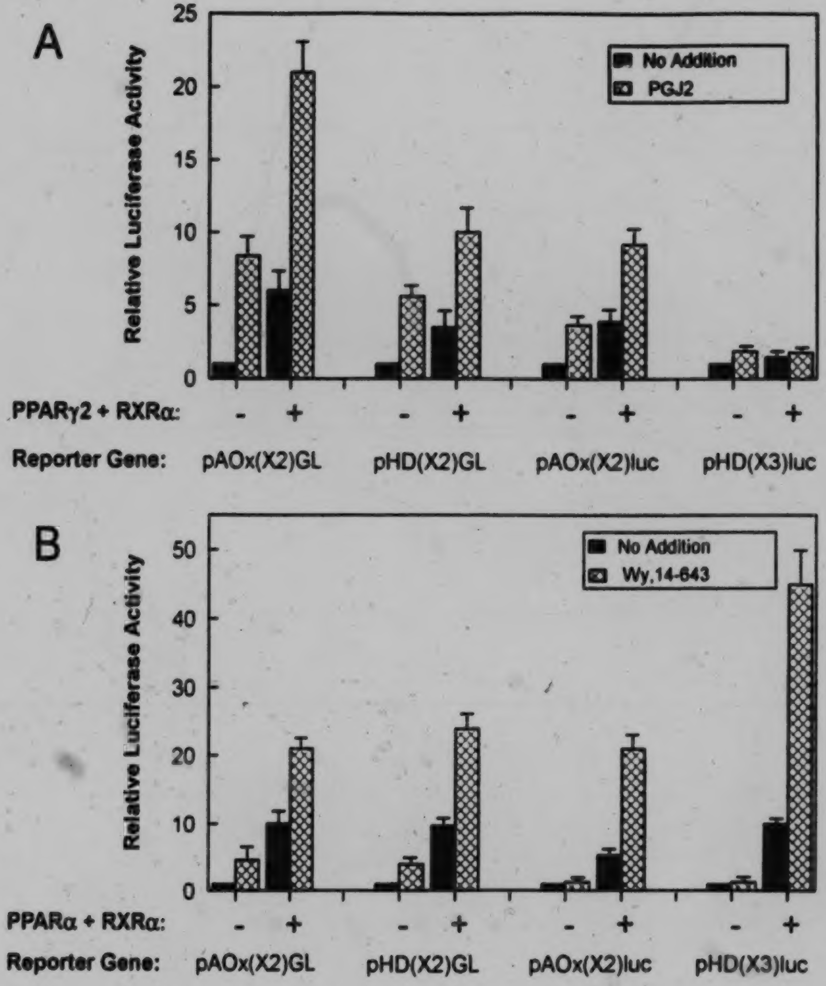


Figure 7-4*. PPARγ2 differentially activates transcription from the AOx-PPRE and HD-PPRE reporter genes in mammalian cells. BSC40 cells were cotransfected with the indicated luciferase reporter genes along with the expression plasmids for PPARγ2 and RXRα (A) or PPARα and RXRα (B) in the presence, or absence, of 15d-PGJ2 or Wy-14,643, as indicated. Luciferase activity was measured 48 h post-transfection. The values shown are averages (±SEM) from three independent transfections carried out in duplicate and normalized to the values obtained with the corresponding reporter plasmid alone in the absence of ligand (taken as 1).

* *The data for this figure were contributed by John Hunter.*

PPRE-dependent and likely results from the presence of endogenous PPAR in mammalian cells (Tontonoz *et al.*, 1994). pHD(X2)GL was also induced by PPARγ2/RXRα, both in the presence, or absence, of 15d-PGJ2; however, the level of induction and absolute activity were less than that observed with pAOx(X2)GL (Fig. 7-4, A). For comparison, experiments were carried out with PPARα and the peroxisome proliferator Wy-14,643, a potent activator of the PPARα subtype (Fig. 7-4, B). In the absence of proliferator, PPARα-mediated induction was comparable with both response elements (10-fold over control levels). The addition of Wy-14,643 resulted in a further 2-fold stimulation of activity from the AOx-PPRE and a slightly higher, but reproducible, 2.5-fold stimulation from the HD-PPRE, when compared to the activity of the reporter gene alone in the presence of proliferator.

To exclude promoter context-dependent effects, transfections were conducted with the minimal promoter from the carbamoyl phosphate synthetase gene. As shown in Figure 7-4, A, PPARγ2/RXRα in the presence of 15d-PGJ2 induced expression of pAOx(X2)*luc* 2.5-fold over the reporter gene construct alone, consistent with results obtained with pAOx(X2)GL. In contrast, PPARγ2/RXRα was unable to induce expression of pHD(X3)*luc* expression over background levels, even though this reporter construct contained three copies of the HD-PPRE. Activities of both the pHD(X3)*luc* and the pAOx(X2)*luc* reporter gene constructs were efficiently induced by PPARα/RXRα (Fig. 7-4, B), consistent with previous findings (Marcus *et al.*, 1993). The higher activity observed with pHD(X3)*luc* compared to pAOx(X2)*luc* is probably due, in part, to the fact that the former construct contains three copies of a PPRE, while the latter construct only contains two copies of a PPRE. These findings demonstrate a PPARγ2 subtype-dependent preference for the AOx-PPRE versus the HD-PPRE in mammalian cells, as observed in yeast. In comparison, PPARα, which showed a strong preference for the HD-PPRE in yeast, was able to efficiently activate transcription via both the HD-PPRE and the AOx-PPRE in mammalian cells.

7.4 Discussion

PPARα/RXRα, expressed from either high or low copy expressions vectors, activated transcription from the HD-PPRE more greatly than from the AOx-PPRE. The response element preference observed in yeast was not as dramatic as in mammalian cells, where we found that both the AOx- and HD-PPRE reporter genes were activated to comparable levels by both PPAR subtypes. It is possible that in transient transfection assays in mammalian cells, PPARα is expressed at levels sufficient for efficient activation of both elements. Alternatively, strong activation of the AOx-PPRE by PPARα in mammalian cells may be due to the presence of positively acting cofactors not present in yeast. A potential candidate is the coactivator, SRC-1, which has been recently shown to bind to and stimulate transactivation by PPARα on the AOx-PPRE (Zhu *et al.*, 1996; DiRenzo *et al.*, 1997).

Response-element activation with PPARα in yeast correlated with the *in vitro* DNA binding efficiencies of the PPARα/RXRα heterodimer, thereby providing a mechanistic basis for the observed differences in transactivation levels from the HD- and AOx-PPREs. Stronger activation from the AOx-PPRE by PPARγ2/RXRα was also reflected in greater binding of PPARγ2/RXRα than of PPARα/RXRα to the AOx-PPRE. These findings are in agreement with those showing that PPARs can bind PPREs with different affinities, depending on the sequence of the PPRE and the receptor subtype (Juge-Aubry *et al.*, 1997).

The data presented herein demonstrates for the first time that PPARγ2 functions as a constitutive transcriptional activator in yeast. Activity was not further augmented by the addition of the PPARγ-specific ligand 15d-PGJ2 and/or the RXRα-selective ligand *9-cis* retinoic acid. The lack of response of nuclear hormone receptors to exogenously added activating ligands in yeast is not uncommon and could be due to, for example, poor uptake of ligand or lack of regulatory components in yeast (Henry *et al.*, 1995; Butt and Walfish, 1996). In this respect, it has been shown that the nuclear hormone receptor-interacting protein, GRIP-1, can restore or augment the ligand-responsiveness of some nuclear hormone receptors in yeast (Walfish *et al.*, 1997). PPARγ2 displayed several properties distinct from those of PPARα. The AOx-PPRE served as a more robust response element for activation by PPARγ2 than did the HD-PPRE, particularly when low copy expression vectors were used. This response element difference was also observed in mammalian cells, where PPARγ2-mediated activation was more efficient with reporter genes containing the AOx-PPRE rather than the HD-PPRE. In contrast to what was observed with PPARα, the difference in response element-dependent transactivation potential of PPARγ2 did not correlate with intrinsic differences in the ability of PPARγ2/RXRα heterodimers to bind to these elements *in vitro*. Indeed, PPARγ2/RXRα heterodimers bound modestly better to the HD-PPRE than to the AOx-PPRE (Fig. 7-3). These observations suggest that differences in binding affinity cannot by themselves account for the response element differences in transactivation observed *in vivo*. Interestingly, expression of PPARγ2/RXRα from high copy vectors did not further stimulate activation from the AOx-PPRE but dramatically increased activation from the HD-PPRE. This suggests that weak activation of the HD-PPRE reporter gene can be compensated by increased levels of PPARγ2, and that threshold levels for efficient activation of the two PPREs in yeast are different. This result contrasts with what is observed with PPARα, where increased activation of both reporter genes by PPARα/RXRα directly correlated with increased expression of the receptors. These findings indicate that response element-specific activation patterns can be differentially affected by PPAR abundance in a subtype-dependent manner. Surprisingly, PPARγ2 was capable of activating transcription from the HD-PPRE to some extent in the absence of RXRα. The basis for this activation is unknown, but the activation was specific, since it was not observed with the AOx-PPRE reporter gene or with PPARα. It may be that PPARγ2 can bind to the HD-PPRE independently of RXRα and activate transcription in yeast, or that some yeast factor substitutes for RXRα to allow complex formation. However, the second possibility seems unlikely, since PPARγ2 in yeast extracts was unable to bind PPREs in the absence of RXRα. The mechanism by which PPARs transactivate in yeast are unknown, and it may be possible that the observed RXRα-independent activation observed with PPARγ2 is peculiar to this organism.

Another distinctive feature of PPARγ2 is related to its cooperative interaction with RXRα. As expected, RXRα cooperated with PPARα to synergistically activate transcription from both the AOx- and HD-PPREs, and with PPARγ2 from the AOx-PPRE. However, with the HD-PPRE, coexpression of RXRα increased activation less than 2-fold, as compared to PPARγ2 alone. This contrasts with a 10 to 20-fold increase in transcription observed with the AOx-PPRE reporter gene when PPARγ2 and RXRα were coexpressed. Thus, while PPARγ2/RXRα heterodimers bind efficiently and cooperatively to both the AOx- and HD-PPREs, much stronger cooperative transactivation is observed on the AOx-PPRE. It is possible that heterodimeric partners are more effective in promoting transactivation via the HD-PPRE. Recent findings have shown that the RXRγ isoform is a more effective heterodimeric partner for binding PPARγ to the HD-PPRE, whereas RXRα is more efficient for binding to the AOx-PPRE (Juge-Aubry *et al.*, 1997).

7.5 Summary

The underlying mechanisms that govern response-element and subtype-dependent differences in PPAR activity in mammalian cells are unknown, but likely relate in part to differences in PPREs. The AOx-PPRE is a simple DR1 array, while the HD-PPRE is more complex, consisting of two DR1 arrays that overlap to form a DR2 repeat (Chu *et al.*, 1995; Horwitz *et al.*, 1996). These structural differences may dictate protein/DNA complex formation and stability, interactions with multiple cofactors and, perhaps, promote conformational changes in bound receptors. For example, evidence indicates that is some cases, recruitment of cofactors to receptors may be governed by allosteric effects that are manifested in a response element- and receptor-dependent manner (DiRenzo *et al.*, 1997).

Thus, the transactivation potential of a given PPAR/RXR heterodimer may be related to PPRE-dependent conformational changes that mask, or unmask, functional domains. In this respect, PPARγ2 has been shown to efficiently interact with the corepressors NCoR and SMRT in solution, but not when bound to the AOx-PPRE as a heterodimeric complex with RXRα (DiRenzo *et al.*, 1997; Zamir *et al.*, 1997).

The results presented herein demonstrate PPRE- and receptor subtype-dependent differences in the capacity of two members of the PPAR subfamily to activate transcription. In some cases, transactivation potential is governed by PPAR abundance and/or differing affinity for particular response elements. However, as seen with PPARγ2, additional parameters appear to enable PPAR subtypes to differentially utilize diverse PPREs.

CHAPTER 8

General Discussion

8.1 General Discussion

The nuclear hormone receptor superfamily is the largest group of eucaryotic transcription factors that regulate development and metabolism through the control of gene expression (Weatherman *et al.*, 1999). PPARs are ligand-activated transcription factors that regulate the expression of genes involved in a plethora of metabolic processes including lipid and glucose metabolism, adipogenesis, and inflammation. This subfamily of nuclear hormone receptors has far-reaching implications for a variety of pathophysiological disease states such as obesity, diabetes and atherosclerosis. Interestingly, obesity is a major risk factor for insulin resistance and cardiovascular disease and is increasing in prevalence in society (Bray *et al.*, 2000). In the United States alone, one-third of the population is now obese (Greenway *et al.*, 1999). These disease states are invariably related to dysfunctional lipid metabolism and homeostasis. Since PPARs are critical sensors of fatty acids level and implicated in peroxisomal fatty acid β-oxidation, they are excellent candidate drug targets. The fibrate family of drugs, the insulin-sensitizing thiazolidinediones, and the nonsteroidal anti-inflammatory drugs are examples of therapeutics that are currently used to circumvent lipid-related disease. A comprehensive understanding of the molecular mechanisms involved in PPAR-mediated gene expression and their putative ligands is required for the continued discovery of effective pharmacological agents for the treatment of human disease stemming from dysfunctional lipid metabolism.

This thesis presents new findings related to PPAR activity with respect to the expression of the genes encoding the first two enzymes of peroxisomal β-oxidation, acyl-

CoA oxidase (AOx) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD). In particular, this thesis expands on the general concepts of transcriptional regulation by illustrating the effects of the TRα, RevErbα, CARβ, and SHP nuclear hormone receptors on PPARα-mediated transcription from the AOx- and HD-PPREs. Furthermore, a comparison of the transactivation potential of two PPAR subtypes, α and γ, has also been demonstrated from both PPREs.

8.2 Convergence of the Thyroid Hormone and Peroxisome Proliferator Signaling Pathways

Peroxisome proliferators and tri-iodothyronine (T3) are important regulators of genes involved in a variety of cellular processes including lipid metabolism, adipocyte differentiation, and energy balance (Chu *et al.*, 1995; Xiong *et al.*, 1998). Both peroxisome proliferators and T3 have been found to act either synergistically or in opposing manners on specific genes involved in lipid metabolism, by acting through a receptor-based mechanism involving members of the nuclear hormone receptor superfamily, namely PPARα and TRα.

Chapter 3 provides evidence for the modulatory role of TRα on PPARα/RXRα-mediated gene transcription from the AOx-PPRE. We have demonstrated that TRα binds to the AOx-PPRE as a monomer and as a heterodimer with RXRα. Furthermore, in transient transfections, TRα augmented PPARα/RXRα-mediated transactivation from the AOx-PPRE in the presence and absence of the peroxisome proliferator, Wy-14,643. In contrast, TRα dramatically repressed transactivation by PPARα/RXRα in the presence of T3. Chu *et al.* (1995) carried out similar studies and demonstrated that PPARα/RXRα-mediated transcription of the genes encoding AOx and HD was repressed by T3. However, in contrast to our observations, they illustrated that TRα repressed PPARα/RXRα transactivation from both the AOx- and HD-PPREs in the presence of peroxisome proliferators. Miyamoto *et al.* (1997) also published results similar to those of Chu and colleagues, but suggested that T3 had no effect on repression. The discrepancy between these findings and our findings presented herein can be explained, in part, by inherent differences between the cell types used in transient transfections. We used a BSC40 African green monkey kidney epithelial cell line, whereas others have used the African green monkey CV-1 kidney fibroblast and the CV-1-derived SV40 transformed COS-1 cell line. The rat hepatoma epithelial H4IIEC3 cell lines have also been used. It is conceivable that cell-specific factors such as endogenous nuclear hormone receptors, coactivators, and corepressors differ between these cell lines. A careful examination of endogenous cofactors in these cell lines would shed light on the observed differences, especially with respect to RXRα levels. We and others (Chu *et al.*, 1995; Juge-Aubry, 1995) argue that the modulatory activity of TRα on PPARα signaling arises from the squelching of RXRα. Therefore, if competition at the level of limiting amounts of RXRα protein determine whether TRα will act positively or negatively, it may be that BSC40 cells contain higher amounts of endogenous RXRα relative to the other cell lines, overcoming any potential inhibitory effects that would otherwise be the case if RXRα were limiting.

Another mechanism by which TRα exerts its effects is by competition for binding to the PPRE. TRα can function as a monomer, homodimer or heterodimer (Desvergne, 1994; Ikeda *et al.*, 1994; Tsai and O'Malley, 1994). We observed that TRα bound to the AOx-PPRE as a monomer and as a heterodimer with RXRα. It has been argued that since a mutation in the DNA binding domain of TRα abrogates the inhibitory action of TRα on transcription from the AOx-PPRE, competition for DNA binding is a requisite for TRα-mediated repression of transcription (Miyamoto *et al.*, 1997). While this may be plausible, it was noted that some inhibition of transcription still occurred with the DNA binding mutant, which may have formed nonbinding heterodimers with PPARα *in vivo* (Miyamoto *et al.*, 1997). Indeed, PPAR/TR heterodimers have been reported to form in solution (Bogazzi *et al.*, 1994). This may account for the reduced PPARα/RXRα binding to the AOx-PPRE we observed *in vitro*, and could explain the repressive effects of TRα *in vivo* in conditions of limiting RXRα. It would be interesting to see how the TRα DNA binding mutant functions in our mammalian expression studies to see if a decrease in activity is similarly observed. Nagaya and coworkers (1992) have suggested that T3 reduces the formation of TRα homodimers, while increasing the formation of TRα/RXRα heterodimers. We observed potent inhibition of PPARα/RXRα-mediated transactivation by TRα in the presence of T3, supporting our hypothesis that sequestration of RXRα by TRα causes transcriptional repression. Indeed, sequestration of RXRα has also been implicated in inhibition of PPARα-mediated transcription by RARs (Imakado *et al.*, 1995).

Transcriptional repression can also result from the sequestration of other cellular cofactors such as the SMRT, NCoR, and SUNCoR corepressors. In the absence of ligand, TRs act as potent transcriptional silencers (Baniahmad *et al.*, 1993; Burcin *et al.*, 1994;

Nawaz *et al.*, 1995; Zhang *et al.*, 1999) through their interaction with corepressor molecules (Baniahmad *et al.*, 1995; Chen and Evans, 1995; Sande and Privalsky, 1996). In this manner, TRα may sequester corepressors that compete for binding to PPARα and RXRα (Chen and Evans, 1995; Kurokawa *et al.*, 1995; Lee *et al.*, 1995). In doing so, the trapping of corepressors by TRα may concomitantly promote the association of liganded PPARα/RXRα heterodimers with coactivators, generating an overall net increase in transactivation by PPARα/RXRα. Transient transfection studies coexpressing coactivator and corepressor proteins may provide additional insight into the modulatory effects observed. If such is the case, point mutations in TRα, PPARα, and RXRα that abrogate coactivator and corepressor interactions would establish the *in vivo* dependency of these interactions for the modulatory role of TRα on PPARα/RXRα-mediated gene transcription.

It has been recently suggested that the H12 α-helix of RXRα may be critical in TR-mediated transcriptional repression (Zhang *et al.*, 1999). It has been proposed that the H12 α-helix imposes steric hindrance on the RXRα conformation, concealing its corepressor interaction surface. However, docking of TRs with RXRα reorients the H12 α-helix, resulting in a conformational state that exposes the interaction surfaces, enabling RXRα (and TRs) to bind corepressor molecules. Considering this, it is conceivable that titration of RXRα by TRα increases the association with corepressors, resulting in a larger pool of active PPARα/RXRα heterodimers that can potentiate transcription from the AOx-PPRE.

Ligand-dependent receptor activity is also differentially modulated by the number of copies of the response element contained in a reporter construct. Olson *et al.* (1997) have shown that TRs act as transcriptional repressors with reporter constructs containing a single copy of a DR4 element, but function as activators from response elements with three copies. Our *in vivo* studies used a synthetic reporter construct containing two copies of the AOx-PPRE in tandem. In this context, it may be that TRα homodimers or TRα/RXRα heterodimers co-occupy the AOx-PPRE simultaneously and synergistically enhance transactivation *in vivo*. Studies conducted on natural promoter elements may be better suited for understanding the true *in vivo* mechanisms of action of nuclear hormone receptors.

It is clear that crosstalk between TRα, PPARα, and RXRα receptor pathways is a complex mechanism governed by many regulatory parameters. A closer examination of the roles played by factors such as SMRT, NCoR, SUNCoR, SRC-1, CBP/p300 as well as the DRIP/TRAP complexes will be of interest in determining the requirements of such cofactors in regulating transcription from the AOx-PPRE. Indeed, there are a number of receptor-specific cofactors utilized by TRα such as TRAP, Trip1, SUNCoR (Lee *et al.*, 1995; Tong, *et al.*, 1996; Zamir *et al.*, 1997) and by PPARα such as PRIP and PBP (Gelman *et al.*, 1999; Zhu *et al.*, 2000) . The role these and other cofactors play will no doubt influence the transcriptional activity of these nuclear receptors. The presence of endogenous receptors in mammalian cells can complicate the analysis of gene transcription by nuclear hormone receptors *in vivo*. Yeast, which lack endogenous nuclear hormone receptors and associated cofactors (McDonell *et al.*, 1989; 1991; Marcus *et al.*, 1996; Butt and Walfish, 1996), would provide an excellent model system in which to carry out parallel studies examining PPARα/RXRα-mediated gene transcription from the AOx-PPRE.

8.3 Signaling Crosstalk Between PPARα and RevErbα

RevErbα is a liver-expressed orphan nuclear receptor that is part of a novel subclass of receptors that bind to target response elements as monomers (Scearce *et al.*, 1993; Carlberg *et al.*, 1994; Giguère *et al.*, 1994; Retnakaran *et al.*, 1994; Harding and Lazar, 1995; Adelmant *et al.*, 1996). Monomeric RevErbα binds to consensus half-sites that are conserved in the HD-PPRE. We have demonstrated that RevErbα binds to the second half-site (site II) of the HD-PPRE *in vitro* and functions to antagonize PPARα/RXRα-mediated gene transcription *in vivo*. Interestingly, site II has been previously demonstrated to be important for the binding of other nuclear hormone receptors such as HNF-4 (Winrow *et al.*, 1994), COUP-TF (Miyata *et al.*, 1993), RZRα (Winrow *et al.*, 1998), and CARβ (Chapter 5). Unlike the AOx-PPRE, the HD-PPRE is a complex response element comprised of two tandem DR1 arrays separated by two nucleotides, resulting in the formation of a juxtaposed DR2. Essentially, the HD-PPRE houses four hexameric direct repeats making it amenable to the binding of multiple nuclear hormone receptors and enabling crosstalk among various signaling pathways.

It has been proposed that PPARα/RXRα cooperatively binds to both DR1 elements *in vivo* (Chu *et al.*, 1995), yielding maximum transactivation from the HD-PPRE. Although we do not observe this interaction in our *in vitro* binding assays, this dual complex may still form *in vivo*, perhaps stabilized through the association of cellular coactivators such as SRC-1, CBP/p300, and PBP (Hörlein *et al.*, 1995; Chen and Evans, 1995; DiRenzo *et al.*, 1997; Dowell *et al.*, 1997). This would allow RevErbα to displace the potential PPARα/RXRα heterodimers from the proximal DR1 site, thereby causing repression.

Besides causing repression via the HD-PPRE, RevErbα has also been shown to repress transcription from the PPRE of the rat apolipoprotein A-1 gene in response to fibrates. Interestingly, fibrates also induce the expression of RevErbα through PPARα activation from a RevErbα DR2 element in the promoter of the human RevErbα gene (Gervois *et al.*, 1999). However, in a negative feedback mechanism, RevErbα subsequently serves to autoregulate its expression by competing for binding to the same RevErbα DR2 element (Adelmant *et al.*, 1996; Vu-Dac *et al.*, 1998; Gervois *et al.*, 1999). Mutations in the RevErbα DR2 site abolishes both PPARα-mediated transactivation and RevErbα-mediated transrepression. These studies support our view that crosstalk between RevErbα and PPARα occurs at the level of DNA binding, whereby PPARα/RXRα and RevErbα monomers compete for binding to the HD-PPRE. This is further substantiated by the lack of transrepression by RevErbα from the AOx-PPRE, to which RevErbα failed to bind *in vitro*. Therefore, DNA binding may define a general mechanism of transrepression of putative targets by RevErbα. For example, since RevErbα expression is increased during adipocyte differentiation (Chawla and Lazar, 1993), it is possible that during this highly sequential and ordered event, RevErbα may downregulate itself and other adipocyte-specific genes such as the leptin gene or even the PPARγ gene. Moreover, since RevErbα expression is controlled by fibrates and represses HD and apolipoprotein A-1 gene expression, RevErbα may play a critical role in overall lipid metabolism and potentially contribute to the onset of atherosclerosis (Gervois *et al.*, 1999). Therefore, it is essential to determine if RevErbα plays a general role as a negative modulator in lipid and lipoprotein metabolism by identifying other target genes that may be regulated by this receptor, or if expression of the RevErbα gene itself is disregulated. Gene chip technology can readily aid in this pursuit by comparing global gene expression patterns in normal and peroxisome proliferator-induced states.

Incidently, the mechanism of RevErbα-mediated transcriptional repression may also arise from interactions with coactivators and corepressors. RevErbα lacks a conserved AF2 domain which is normally used for interactions with coactivators. Its absence would suggest the inability of RevErbα to bind coactivators, illustrating a potential mechanism by which it acts as a constitutive transcriptional repressor. However, no studies have been conducted demonstrating a lack of interaction with coactivators. The lack of a physical AF2 domain does not preclude the possibility that RevErbα contains novel activation domains (non-AF2) that can mediate interactions with coactivators. RevErbα may use its amino-terminal AF1 domain in a manner similar to estrogen receptor β and steroid factor 1 to interact with coactivators (Tremblay *et al.* 1999; Hammer *et al.*, 1999).

As mentioned earlier, transrepression can be mediated by corepressors. It may be that RevErbα has a strong affinity for corepressor molecules such as NCoR and SUNCoR which may circumvent the effects of ligand-activated PPARα/RXRα heterodimers *in vivo* (Zamir *et al.*, 1996). Similarly, recruitment of corepressors by RevErb may form a large, multimeric complex on the HD-PPRE, causing spatial interference that displaces PPARα/RXRα heterodimers from the HD-PPRE or prevents their access to the HD-

PPRE. SUNCoR interacts with NCoR and SMRT *in vitro* (Zamir *et al.*, 1997), which may act to stabilize a RevErbα repression complex with additional proteins such as Sin3A and HDAC-1. Although both NCoR and SUNCoR have been demonstrated to interact with RevErbα, this interaction has been shown to occur when RevErbα is bound to a RevErbα DR2 site. It would be useful to verify this interaction by monomeric RevErbα as well. Yeast two-hybrid screens and two dimensional gel electrophoresis can be useful to isolate and identify associated cofactors that contribute towards the repressive activity of RevErbα.

Our results suggest that the HD gene serves as a specific target for RevErbα, which acts as a negative regulator of PPARα/RXRα-mediated transactivation.

8.4 Integration of CARβ, PPARα, and RXRα Signaling Pathways

The orphan nuclear receptor CARβ binds DNA as a heterodimer with RXRα and activates gene transcription from a subset of retinoic acid response elements (RARE) in a constitutive manner (Baes *et al.*, 1994; Choi *et al.*, 1997; Forman *et al.*, 1998). Similarly, we have demonstrated that CARβ/RXRα heterodimers occupy the DR2 motif of the HD-PPRE and stimulate transcription from this PPRE in a ligand-independent manner. CARβ/RXRα has also been shown to bind to and transactivate from the steroid/rifampicin-responsive ER6 element of human *CYP3A4* and the phenobarbital-inducible DR4 motif of the phenobarbital response element module (PBREM) located in the promoter of the *CYP2B* gene (Honkakoski and Negishi, 1998; Sueyoshi *et al.*, 1999). This display of binding promiscuity is typical of many nuclear hormone receptors. It is well known that binding specificity is influenced by the sequence, spacing and orientation of half-sites on a response element (Umesono and Evans, 1989; Umesono *et al.*, 1991; Lefstin and Yamamoto, 1998). In the context of PPREs, it may be that DR2 elements provide the minimum spatial distance for the efficient binding of CARβ/RXRα heterodimers, since they failed to form a complex on the DR1 element of the AOx-PPRE. However, the binding polarity of a heterodimeric complex may also influence the binding sensitivity to a given response element. For heterodimers of RXRα, RXRα is routinely observed to bind to the 3' TGACCT half-site (Kurokawa *et al.*, 1994; Rastinejad *et al.*, 1995). Consistent with this, we have inferred from our *in vitro* data that RXRα occupies the distal half site of the juxtaposed DR2 motif of the HD-PPRE, while CARβ occupies the 5' TGACCT motif.

Differentially spaced half-sites can also produce distinct transcriptional responses from nuclear hormone receptors (Lefstin and Yamamoto, 1998). For example, RAR/RXR heterodimers activate transcription from DR5 elements in a ligand-dependent manner, but fails to do so from DR1 elements. This has been attributed to the inability to release the NCoR repressor protein (Kurokawa *et al.*, 1995). One can envision an analogous, albeit inverse mechanism, for ligand-dependent CARβ/RXRα heterodimers. The steroid androstanol ligands have been shown to potently deactivate the constitutive activity of CARβ by dissociating the SRC-1 coactivator (Forman *et al.*, 1998; Sueyoshi *et al.*, 1999). We observed only a modest reduction of ligand-independent activity by CARβ/RXRα heterodimers in the presence of androstanol. This observation has led us to hypothesize that allosteric constraints arising from the HD-PPRE DR2 element induces a conformational state with CARβ/RXRα that makes the release of SRC-1 inefficient or, conversely, prevents the exposure of an interaction surface for the recruitment of corepressors. Additionally, endogenous levels of nuclear hormone receptors, cofactors or other cell-specific proteins may have contributed to the diminished effects of the androstanol ligands in the BSC40 cell line, since the same effects were seen for transactivation from the β-RARE, *in vivo*. As part of this explanation, we suggest that endogenous levels of active PPARα/RXRα heterodimers overcome the effects of androstanol deactivation by promoting transcription from the HD-PPRE and β-RARE.

In the presence of PPARα/RXRα, CARβ had no effect on transcription from the HD-PPRE, whereas it antagonized transactivation from the AOx-PPRE. Because CARβ/RXRα heterodimers form a complex on the HD-PPRE but not on the AOx-PPRE, we suggest CARβ sequesters RXRα, decreasing the amount of transcriptionally active PPARα/RXRα heterodimers from the AOx-PPRE. As previously discussed, sequestration may also involve other cellular cofactors as well. This can be verified by conducting coimmunoprecipitation studies with CARβ to see if various transcriptional components are shared with PPARα and/or RXRα.

Recently, the phenobarbital-like compound 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) has been demonstrated to be a CARβ ligand which, in contrast to androstanol, potentiates CARβ transactivity (Sueyoshi *et al.*, 1999; Tzameli *et al.*, 2000). Furthermore, TCPOBOP can overcome the inhibitory effects of androstanol ligands, suggesting it acts as a potent, competitive ligand for CARβ *in vivo*. The fact that a xenobiotic compound can activate CARβ suggests that other members of the peroxisome proliferator class of xenobiotic compounds may also serve as putative ligands for CARβ. Current research shows that xenobiotics induce the transcription of the *CYP2B*, *CYP3A*, and *CYP4A* genes by CARβ, PXR, and PPARα, respectively (Lee *et al.*, 1995; Huss *et al.*, 1996; Johnson *et al.*, 1996; Honkakoski and Negishi, 1998; Kliewer *et al.*, 1998; Sueyoshi *et al.*, 1999; Strom *et al.*, 1996). Therefore, it is tempting to speculate whether CARβ is integrated with other nuclear hormone receptor signaling pathways, most notably with PPARα, in response to similar classes of ligands. Secondly, the induction of the cytochrome P450 series of genes implies that both CARβ and PPARα may coinduce the metabolism of steroids, lipids, fatty acids and other xenobiotics to produce potential ligands not only for each other, but for other nuclear hormone receptors (Sueyoshi *et al.*, 1999). As an example, the pesticide methoxychlor, which is converted to an estrogen-based compound by the *CYP2B* enzyme (Bulger *et al.*, 1985), also activates CARβ and *CYP2B* expression (Sueyoshi *et al.*, 1999). Therefore, CARβ could be involved in increasing the availability of putative ligands for estrogen receptors. An in-depth screen of gene activation using a comprehensive chemical library provides a means of correlating gene expression patterns with receptor activation and deactivation. This may also prove useful in the design of pharmacologics that lack receptor cross-reactivity and decreased drug-drug interactions.

Recently, Moore and coworkers (2000) have compared the effects of various xenobiotics on CARβ-mediated CYP expression. Using fluorescence resonance energy transfer (FRET) ligand-sensing assays (Parks *et al.*, 1999) to measure CARβ/SRC-1 interactions, as well as radioligand binding competition assays, they established that androstanol, 5β-pregnane-3,20-dione (a PXR ligand), and the antimycotic agent clotrimazole, all bind CARβ (Moore *et al.*, 2000). Nevertheless, ligands such as androstanol and 5β-pregnane-3,20-dione may not be physiogically relevant, since superphysiological concentrations are required to mediate their effects. This raises the possibility that *bona fide* steroid ligands remain to be found, including intermediate metabolites formed by one of many CYP enzymes (Waxman, 1999). Moreover, Moore *et al.* (2000) have also demonstrated species-specific responses to various xenobiotics with the human and mouse CARβ isoforms. These may be the result of post-translational modifications, differences in spatial expression patterns, or both. Indeed, phosphorylation has been shown to result in the targeting of nuclear hormone receptors, including CARβ, from the cytosol to the nucleus (Kawamoto *et al.*, 1999). Other possibilities may still account for species- and ligand-specific responses, meriting the continued analysis of the molecular mechanism of action of CARβ with PPARα and RXRα, and with other members of the nuclear hormone receptor superfamily. Future studies may eventually uncover the reactivity of different xenobiotics with CARβ, PPARα, and RXRα and their modulation of not only CYP and HD gene expression, but the expression of other target genes as well. These studies can then be extended to examine the activation potential of xenobiotics between receptor subtypes such as PPARγ and PPARδ. Targeted disruption of the CARβ gene may provide additional insight into the physiology of this receptor. High throughput binding assays and cell-based transfections will no doubt help in unravelling the highly integrated signaling pathways of nuclear hormone receptors.

8.5 Modulation of Transcription From the AOx- and HD-PPREs by SHP

The mechanism of action of SHP offers some challenges to the basic concepts of transcriptional regulation by nuclear hormone receptors. Our results indicate that SHP positively and negatively regulates PPARα/RXRα-mediated transcription from the HD-PPRE and AOx-PPRE, respectively. Notwithstanding our incomplete analysis, we have proposed a number of mechanisms to account for the modulatory role of SHP from PPREs. (1) Structural differences between the AOx- and HD-PPREs may influence protein/DNA formation and stability by increasing or decreasing steric hindrance of bound receptor complexes (DiRenzo *et al.*, 1997). These allosteric effects may position PPARα/RXRα heterodimers in a conformational state that allows for the interaction of SHP, which subsequently represses transcription via its carboxy-terminal repressor domain. Binding of SHP may also prevent the association of coactivator molecules. However, in the context of the HD-PPRE, using the LXXLL-related motifs, SHP may function as a novel coactivator-like protein, potentiating transcription. If this is true, it is likely that SHP would be associated with other classical coactivators like CBP/p300 or the DRIP/TRAP complexes. This could be verified by isolating such factors by coimmunoprecipitation, yeast two-hybrid screens or epitope-tagged *in vitro* binding assays. (2) Although lacking a typical DNA binding domain, SHP may be capable of binding to DNA *in vivo* using novel structural domains. Both the AOx- and HD-PPREs may direct the specificity of binding of SHP monomers, homodimers or heterodimers with either RXRα or PPARα. It has been noted that the HD-PPRE contains a series of potential binding sites for nuclear hormone receptors. These sites increase the possibility that SHP may stabilize an existing PPARα/RXRα heterodimer on this PPRE or bind to this element together with PPARα and RXRα, individually or synergistically to activate transcription. (3) Like TRα and CARβ, SHP may sequester PPARα and RXRα from each other in a response-element specific manner that differentially modulates transcription. In the context of the AOx-PPRE, SHP may form inactive, nonbinding heterodimers. This is supported by our observations from gel electromobility shift assays. Using combinations of *in vitro* translated SHP, PPARα, and RXRα, no heterodimeric complex with SHP is observed to bind either the AOx- or HD-PPRE. (4) Seol and colleagues (1997) have been unable to detect any interaction of SHP with NCoR in either yeast or mammalian two-hybrid systems. However, it remains to be seen whether SHP can interact with the related corepressors SMRT and SUNCoR, or with an as of yet unidentified corepressor to cause transrepression. Alternatively, SHP may interact directly with a histone deacetylase, a Sin-3-like protein, or a component of the basal transcription machinery to give a similar effect (Vidal and Gaber, 1991; Taunton *et al.*, 1996).

In summary, although much research is required to ascertain the overall mechanism of action of SHP, it is likely that the proposed mechanisms of action may not be mutually exclusive. Furthermore, it will be important to establish whether SHP is in any way ligand-activated. The presence of a ligand binding domain suggests that a natural ligand may exist, influencing the activating or repressing potential of SHP.

8.6 Transactivation by PPARs in Yeast

Although mammalian cells are a useful model system with which to examine nuclear hormone receptor function *in vivo*, analyses are complicated by the fact that receptor activity varies between cell types and by the presence of endogenous nuclear hormone receptors, cellular cofactors and physiological ligands. These limitations compromise our ability to truly understand the molecular mechanism of peroxisome proliferator signaling pathways. To circumvent these problems, we used the yeast *Saccharomyces cerevisiae* to explore the mechanism of action of PPARs with PPREs. Yeast lack endogenous factors, including nuclear hormone receptors, and it is likely they lack the metabolic machinery for creating or modifying receptor ligands (Hall *et al.*, 1993). Against this null background, yeast serve as an attractive model system with which to examine nuclear hormone receptor activity and to complement studies in mammalian cells. RXRs, RARs, TRs, ERs, and PPARα have all been functionally expressed in yeast (Garabedian and Yamamoto, 1992; Pham *et al.*, 1992; Hall *et al.*, 1993; Heery *et al.*, 1993; Sande *et al.*, 1994; Marcus *et al.*, 1995; Kephart *et al.*, 1996; Berghöfer-Hochheimer *et al.*, 1997). Furthermore, yeast have been used successfully to isolate and clone nuclear hormone receptor-interacting proteins, as well as to define heterodimeric partners for nuclear receptors (Horowitz *et al.*, 1996). Chapter 7 illustrates how a yeast-based system was used to discriminate between the transcriptional activation potential of PPARα and PPARγ2 on *lacZ* reporter genes containing the AOx- and HD-PPREs. We demonstrated that PPARα/RXRα activated transcription more robustly from the HD-PPRE, while PPARγ2/RXRα preferentially transactivated from the AOx-PPRE. The former result is in agreement with previous findings that the HD-PPRE is a more efficient response element for PPARα/RXRα (Marcus *et al.*, 1995). Similar response element-dependent differences in transactivity were observed in mammalian cells. We speculate that structural differences between PPREs modulate transactivity by stabilizing protein/DNA interactions, leading to the observed differences in transactivity between PPARα/RXRα and PPARγ2/RXRα heterodimers. The binding efficiency of these heterodimers to the PPREs *in vitro*, is in agreement with their transactivation potential in yeast, suggesting that these response elements do impart allosteric effects that govern the receptor-specific activation potential *in vivo*. Furthermore, as the HD-PPRE possesses two tandem DR1 arrays, it may be that either site is particularly suited for binding PPARα/RXRα independently or synergistically (Chu *et al.*, 1995). It has also been noted that PPARγ contains an additional α-helix (H2') (Renaud *et al.*, 1995) that may limit the amount of its flexibility on various response elements. As such, PPARγ may be structurally limited to fully transactivate from the HD-PPRE.

The highly divergent A/B domain of nuclear hormone receptors appears to impart a number of receptor-specific activities. Of interest is the demonstration that the A/B regions of PPARα and PPARγ are modified by phosphorylation (Juge-Aubry *et al.*, 1997; Werman *et al.*, 1997) and that the A/B region of PPARγ can influence ligand binding by the ligand binding domain (Shao *et al.*, 1998). In addition, the PPARα and PPARγ2 AF1 domains display different constitutive transcriptional activating properties when expressed in both yeast and mammalian cells (John Capone, McMaster University, personal communication). Functional analysis of the various PPAR subdomains will be important in determining their receptor-specific activities through inter- and intra-domain communication. To map the specific PPAR domains, deletion and point mutations can be introduced to PPAR domains fused to Gal4 DNA binding domains and tested for transactivity from the AOx- and HD-PPREs. Domain swapping experiments will also help to establish if structural conformations are responsible for the response element-specific transactivation potential of both PPAR subtypes. In addition, modifying the residues at sites I and IV of the HD-PPRE to make it resemble the AOx-PPRE, and *vice-versa*, may reverse the binding efficiencies and transactivation potential of the PPAR/RXRα heterodimers. This would provide additional evidence of whether structural and spatial conformational constraints can be substituted between response elements, thereby confirming response element specificity. These studies can eventually be extended to include the PPARδ subtype and also used to examine the ability to dissociate and recruit corepressors and coactivators, respectively.

Interestingly, the PPARγ2 subtype-dependent preference for the AOx-PPRE observed both in yeast and mammalian cells mirrored the binding efficiencies *in vitro*, while PPARα could efficiently activate transcription from both the AOx- and HD-PPREs (two copies of each PPRE). A number of variables may be at work to account for the strong activation potential of PPARα over PPARγ2 with respect to fold-induction over basal activity, in mammalian cells as compared to yeast cells. Since mammalian cells contain a large complement of cofactors that can interact with nuclear hormone receptors, it is likely that SRC-1, CBP/p300, and other PPARα-specific coactivators potentiate PPARα-dependent transactivation. Indeed, this is true with respect to the GRIP1 coactivator protein. GRIP1 has been shown to markedly restore the ability of nuclear hormone receptors such as TR and RAR to transactivate gene expression in a ligand-dependent manner (Walfish *et al.*, 1997). The same argument could be made for PPARγ2; however, our results indicate otherwise. Coexpression of coactivators with PPAR expression plasmids in yeast would establish the modulatory role of coactivators on PPAR subtype transactivity. Conversely, it may also be that corepressor molecules have a greater affinity for PPARγ2 in BSC40 cells. The structural properties of the PPREs may also produce allosteric effects that govern the efficient recruitment of cofactors to the receptors (DiRenzo *et al.*, 1997). Therefore, the transactivation potential of a given PPAR/RXRα heterodimer may be related to PPRE-dependent conformational changes that expose the interaction surfaces for cofactors. Investigating whether there are PPRE-dependent differences in the ability of PPARs to interact with various receptor coactivators both in whole mammalian cell lysate as well as through reconstitution in yeast would help to establish the role of cofactor-dependent subtype-specific transactivity.

We and others have illustrated that yeast are unresponsive to PPAR activation by peroxisome proliferators (Marcus *et al.*, 1995; Henry *et al.*, 1995). Furthermore, we demonstrated that yeast also failed to respond to 9-*cis* retinoic acid or the PPARγ-specific ligand, 15d-PGJ2. This lack of response may be attributed to inefficient uptake of the exogenous ligand, lack of metabolic machinery to modify the potential ligand to biologically active forms (Hall *et al.*, 1993; Henry *et al.*, 1995; Butt and Walfish, 1996), inactivation of the ligand by yeast-specific metabolism, or exocytosis of the ligand. The last has been demonstrated in yeast, whereby ATP-transport proteins actively export exogenous estrogen ligand (Gilbert *et al.*, 1993; Kralli *et al.*, 1995). However, Olson *et al.* (1997) have suggested that ectopically expressed receptors in yeast lacking corepressors may exist in a constitutively active state, functionally equivalent to liganded receptors. This would imply that such receptors are incapable of enhancing transcription above constitutive levels achieved in the absence of ligand. Furthermore, the lack of corepressors would also eliminate any transcriptional repression arising from the recruitment of histone deacetylase, further rendering these nuclear hormone receptors refractory to exogenous ligands. Therefore, in yeast, these receptors remain in a "mono-active" state unlike in mammalian cells. This hypothesis can be addressed by coexpression of coactivators and corepressors with nuclear hormone receptors in the presence and absence of ligand, in an effort to re-establish the repressed, basal, and activated forms of the receptor *in vivo*. Using yeast two-hybrid analysis, the functional utility of the coactivators SRC-1 and p300 to interact with ligand-activated PPARα has been established (Dowell *et al.*, 1997). This result would suggest that coactivators are needed for full activation, while refuting the notion of inefficient ligand uptake, but not necessarily the need for metabolic conversion.

Allegretto and colleagues have shown that RARγ/RXRγ heterodimers and all RXR subtype homodimers could be activated by 9-*cis* retinoic acid in yeast from DR1- and DR5-containing promoter elements (Allegretto *et al.*, 1993). Similarly, Hall *et al.* (1993) have shown that RXRγ/TRβ could potentiate transcription from a TREpal in the presence of the RXRα ligand. Thus, we were surprised that both PPAR/RXRα heterodimer and RXRα homodimers (transformed alone) could not be similarly activated from the AOx- or HD-PPRE in response to 9-*cis* retinoic acid. However, VDR/RXRβ2 and VDR/RXRγ have also failed to respond to either vitamin D or 9-*cis* retinoic acid in yeast (Berghöfer-

Hochheimer *et al.*, 1997; Jin and Pike, 1996). These different observations are hard to reconcile, but may suggest that transactivation by nuclear hormone receptors in yeast is strongly influenced by the RXR subtype and the specific nucleotide spacing between half sites of direct repeats (Kephart *et al.*, 1996). However, it may still be possible to have a peroxisome proliferative response by PPARs in yeast if the heterodimeric partner is RXRβ or RXRγ. Receptor activation may also require much higher concentrations of ligands in yeast than in mammalian cells, though exceedingly high concentrations may eventually be toxic and physiologically irrelevant.

8.7 Future Considerations

The 70 known nuclear hormone receptors may represent only a third of the actual number of receptors that truly exist. A search of the *Caenorhabditis elegans* genome suggests that there exists approximately 228 proteins containing two-zinc finger structures (Gustaffson, 1999; Clark and Berg, 1998). However, it remains to be determined whether mammalian homologs exist for such putative nuclear receptors. Furthermore, the genes controlled by known nuclear receptors may also be modulated *in vivo* by other regulatory pathways currently unknown to researchers. This may include compensatory or dormant pathways that become activated in the absence of a specific nuclear hormone receptor (Deluca *et al.*, 2000). This may be true for the RXR and PPAR subfamilies which have known, yet enigmatic, receptor subtypes that may influence gene expression in a secondary manner by providing basic metabolic requirements for a cell or organism, especially under disease states. For example, TZD administration in PPARα null mice causes a modest induction of AOx protein concomitant with increases in AOx mRNA levels, suggesting that the AOx-PPRE may be targeted by either PPARγ or PPARδ, which act as surrogate receptors for PPARα. Although hepatic expression of PPARγ and PPARδ is lower than that of PPARα, they may still be capable of functioning in peroxisomal β-oxidation. Conversely, activation of PPARγ-mediated transcription of adipocyte differentiation genes by PPARα has also been demonstrated (Brun *et al.*, 1996). These results would suggest that under permissive conditions, PPARs can mediate the actions of their dominant PPAR subtypes in order to maintain normal metabolic processes.

8.7.1 PPARδ: Putative Biological Functions

Unlike the other PPAR subtypes, there are no high affinity PPARδ-selective ligands available for determining the function of this receptor (Willson and Wahli, 1997). The available data for PPARδ, which suggests putative roles for this receptor in lipid homeostasis, fertility, and cancer (Amri *et al.*, 1993; Lim *et al.*, 1999; He *et al.*, 1999) have mainly relied on correlative associations with specific phenotypes. It remains to be determined whether target genes exist that are exclusively controlled by PPARδ (Peters *et al.*, 2000). As a means of unravelling the mysteries of PPARδ function, Peters *et al.* (2000) have generated PPARδ knock out mice which show defects in myelination in the central nervous system and remain refractory to the administration of nonsteroidal anti-inflammatory drugs. Expression of the proteolipid protein, a myelin-specific protein encoded by a gene containing a PPRE, was unaffected in these mice. This was surprising since PPARδ is the most predominant PPAR subtype expressed in the brain (Braissant and

Wahli, 1998). Thus, it remains to be seen what, if any, putative target genes exist for PPARδ, or whether it simply serves as a receptor that acts by proxy for other PPAR subtypes through some signaling event.

8.7.2 Auxiliary Cofactors

It remains unclear how DRIP/TRAP and ARC/SMCC interface with the p160/CBP/p300/pCAF system. It is postulated that histone acetyl transferase-containing coactivators must first unwind chromatin to allow the DRIP/TRAP and ARC/SMCC complexes to bind to the nuclear receptor/coactivator complex and thereafter recruit RNA polymerase II to activate transcription (Freedman, 1999a and 1999b). The need for histone acetyl transferase activity is supported by the demonstration that DRIP/TRAP and ARC/SMCC complexes fail to activate transcription on naked DNA *in vitro*, but do so in the presence of chromatin (Sun *et al.*, 1998; Boyer *et al.*, 1999; Ito *et al.*, 1999; Kingston, 1999). Interestingly, other transcription factors unrelated to nuclear hormone receptors such as Sp1 can also recruit DRIP/TRAP complexes (Freedman, 1999a and 1999b), suggesting that DRIP/TRAP and ARC/SMCC complexes function as a novel subclass of general auxiliary transcription factors.

Similarly, the role of protein methyl transferases in transcriptional regulation remains unclear. It is possible that CARM1 or similar proteins can modulate PPAR-mediated gene transcription. Rachubinski *et al.* have described an approximately 300 amino acid PPARα-interacting protein isolated from a yeast two-hybrid screen that shows homology to protein methyl transferases (personal communication). Therefore, it seems likely that protein methyltransferases will play an important role in transcriptional regulation by PPARs.

8.7.3 Drug Discovery

Through the process of reverse endocrinology, considerable advances have been made towards a better understanding of the biology and physiology of nuclear hormone receptors. The importance of the nuclear hormone receptors as medicinal targets has caused a fevered rush to screen comprehensive chemical libraries in an effort to find putative ligands that can aid in the rational design of pharmaceutical drugs for the treatment of diseases caused by defects in the biochemical pathways involving nuclear and orphan receptors (Gustafsson, 1999). These studies are complicated by the fact that receptors have multiple subtypes and, in general, exhibit a wide array of cellular functions. Given this fact, it will be prudent to develop drugs that display target efficacy and subtype selectivity.

The search for agonist and antagonist ligands for newly discovered orphan receptors will undoubtedly lead to the discovery of previously unknown signaling pathways regulated by specific receptors and their interactions with novel cofactors. With recent advances in combinatorial chemistry, high throughput screening assays, and functional genomics, a better understanding of the complex mechanisms of nuclear and orphan receptor biology will emerge.

8.7.4 A Model of Transcriptional Regulation by Nuclear Hormone Receptors

The central theme that has been emphasized in this thesis is that transcriptional regulation by nuclear hormone receptors from the PPREs of the AOx and HD genes is a complex and dynamic event. The signaling pathways that converge on the AOx- and HD-PPREs are highly integrated to ensure the correct transcriptional response to physiological stimuli. A model (Fig. 8-1) illustrating the convergence of the various nuclear hormone receptors to act on PPARα-mediated gene transcription from the AOx- and HD-PPREs can be proposed.

The mechanism by which nuclear hormone receptors crosstalk to elicit a transcriptional response is influenced by many variables including (i) response element specificity resulting from the sequence, spacing, and orientation of half-sites; (ii) competition for binding to half-sites; (iii) sequestration of common heterodimerization partners, such as RXRα; (iv) interactions with coactivators, corepressors, and auxiliary cofactors; (v) the availability of activating/deactivating ligands; (vi) post-translational modifications; (vii) relative abundance of nuclear receptors and cofactors; (viii) interactions with the basal transcriptional machinery; (ix) accessibility to DNA via histone acetyl transferase, histone deacetylase, and protein methyltransferase activities; and (x) steric hindrance from protein/protein and protein/DNA interactions.

In keeping with the current model of transrepression and transactivity, unliganded nuclear hormone receptors bound to DNA interact with a series of corepressor molecules that recruit histone deacetylase activity, causing DNA to remain tightly associated with histones. Upon ligand binding, corepressor molecules dissociate from the receptors which,

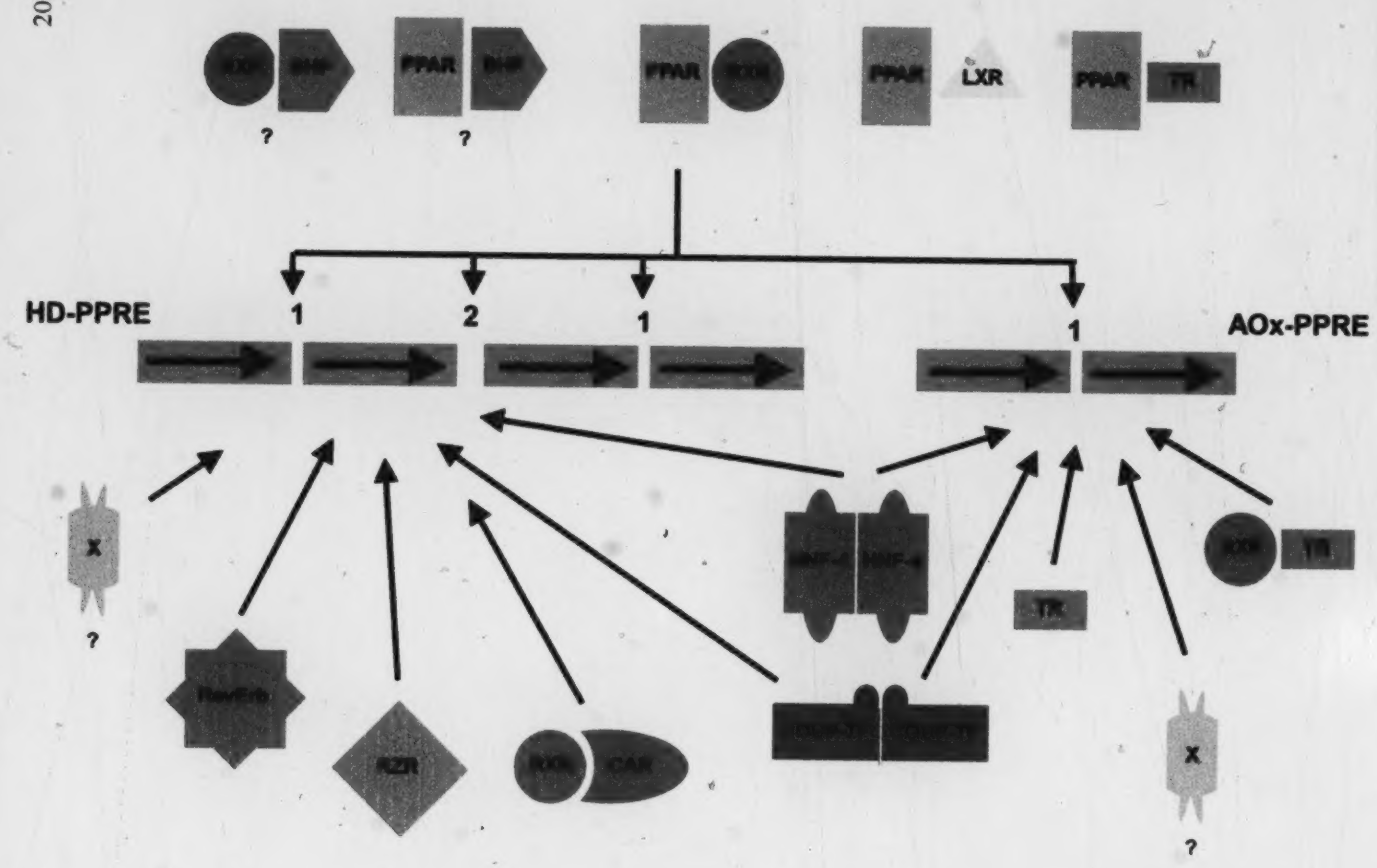

?
PPAR
?
PPAR
PPAR
LXR
PPAR
TR
HD-PPRE
1
2
1
1
AOx-PPRE
X
?
RevErb
RZR
RXR
CAR
HNF-4
HNF-4
TR
TR
X
?

Figure 8-1 Convergence of Nuclear Hormone Receptors on the AOx- and HD-PPREs

in turn, undergo a conformational change promoting the recruitment of classical coactivator molecules, some of which possess histone acetyl transferase activity that remodels chromatin. This enables an association with a large, multisubunit auxiliary protein complex which, in synergy with the coactivators and nuclear receptors, mediates cooperative interactions with the pre-initiation complex (Figure 8-2). This positively acting pathway can then be negatively regulated either by the dissociation or metabolism of the receptor ligand, formation of non-DNA binding receptor complexes, binding of transrepressive receptors, or by the presence of deactivating ligands. The overall effect is a delicately balanced, continuous flux between transcriptional activation and repression.

Because of the dynamic cellular environment, it is difficult to ascertain the dominant transcriptional response in a milieu of cellular receptors, cofactors and endogenous ligands. We propose that ligand binding to a particular nuclear hormone receptor increases the receptors' affinity for cellular pools of receptor cofactors, which ultimately determines the transcriptional response. In this manner, cofactors continuously shuttle from one receptor to another based on "rank-order affinity" to produce the necessary transcriptional response according to the immediate metabolic needs of a cell. This would leave other receptors at a repressed or basal level of activity.

8.8 Conclusion

Previous studies have shown the interplay of COUP-TF, HNF-4, RZRα, and LXRα receptors in modulating PPARα-dependent transactivity. The results presented herein illustrate how four additional members of the nuclear hormone receptor superfamily - TRα,

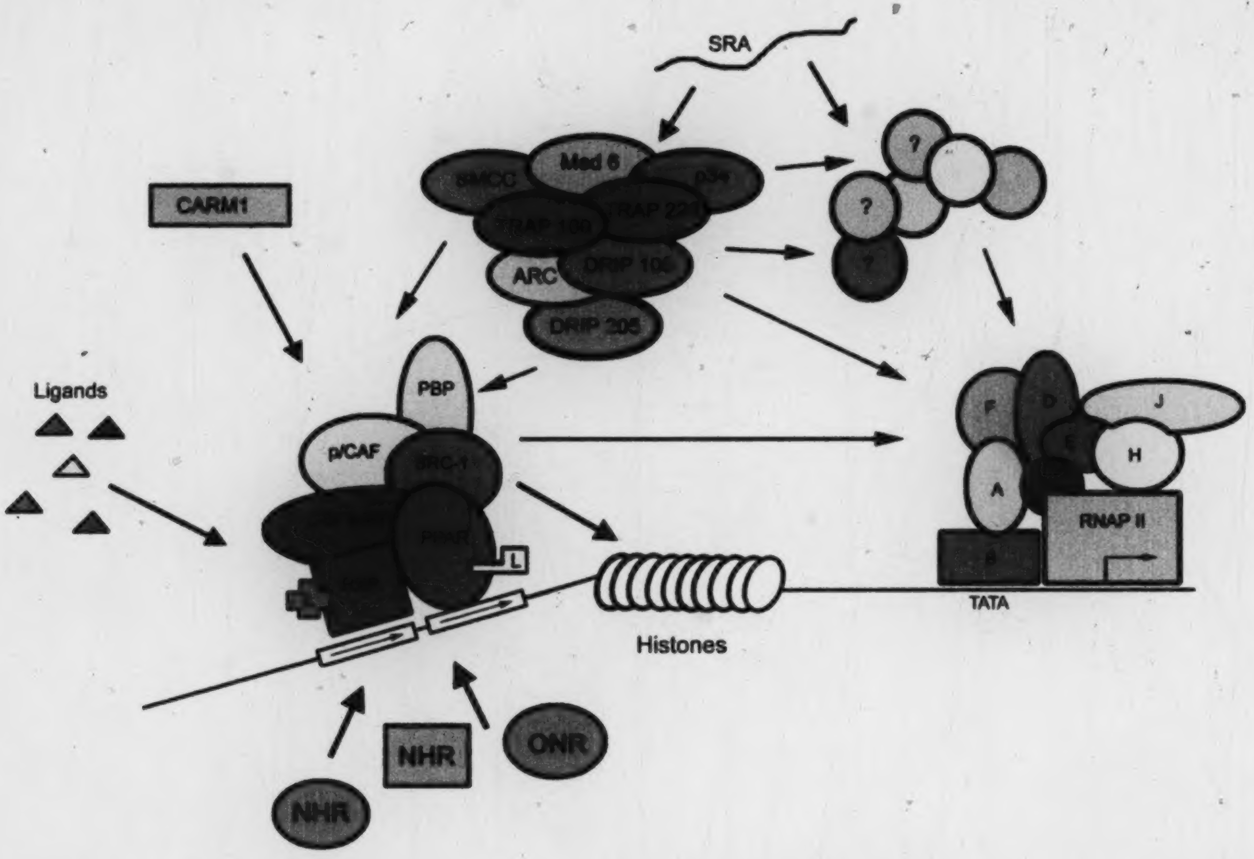

SRA
SMCC
Med 6
TRAP 220
TRAP 100
ARC
DRIP 100
DRIP 205
?
?
?
CARM1
Ligands
PBP
p/CAF
SRC-1
PPAR
L
Histones
F
D
J
E
H
A
B
RNAP II
TATA
NHR
NHR
ONR

Figure 8-2 Overview of factors involved in nuclear hormone receptor-mediated gene transcription

RevErbα, CARβ, and SHP - converge on the AOx- and HD-PPREs and differentially modulate PPARα/RXRα-mediated gene transcription. We have also demonstrated that subtype- and response element-dependent differences also influence transactivation from the two PPREs. This research extends our current knowledge of peroxisome proliferator-mediated gene expression and underscores the complex dimensions involved in regulating the expression of the genes encoding the first two enzymes of the peroxisomal β-oxidation pathway. The research provided in this thesis raises provocative questions that warrant continued efforts to further delineate the intricate molecular mechanisms of PPAR-mediated gene regulation. Establishing the general principles governing the mechanisms of peroxisome proliferator-dependent PPAR activation must be considered in the context of global gene expression in order to fully understand the consequences of peroxisome proliferators in the dynamic environment of a multifunctional eukaryotic organism.

CHAPTER 9

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